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14. ABSTRACT

The overall hypothesis that drives this project is that persistent replication stress generates mutational events in breast epithelial cells that fuel breast cancer (BCa) progression. Our model predicts that a major source of replicative stress in BCa is hypoxia, which stalls active replication forks, and selects for cells that have bypassed this S-phase checkpoint due to mutations in the ATR-hchkl pathway. The specific aims of this project are: (1) to define the role of the ATR checkpoint pathway in hypoxia-induced cytostasis, and (2) to determine whether defects in this checkpoint pathway promotes BCa progression, and confers sensitivity to killing by certain anticancer agents.

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Table of Contents

Cover.. 01

SF 298.. 02

Introduction.. 04

Body.. 04-06

Key Research Accomplishments.. 06

Reportable Outcomes.. 07

Conclusions.. 08

References.. 09

Appendices.. 10-38

INTRODUCTION

This proposal was based on the hypothesis that several prominent breast cancer (BCa) susceptibility genes encode proteins that function in replication stress response pathways in mammalian cells. Examples of such S-phase surveillance-associated genes include *BRCA1*, *BRCA2*, *ATM*, *hCHK1*, and *hCHK2*. The striking links between the occurrence of germ-line or sporadic loss of function mutations in these genes suggests that the cells residing in mammary tissue are particularly dependent on the replication stress response to maintain genomic stability, and, to prevent these cells from embarking on the path to malignancy Defects in the S-phase checkpoint machinery may also contribute to malignant progression in early-stage breast tumors. In a report that served as the background for this proposal, we demonstrated that deep hypoxia (0.1% O_2) caused cancer cells to arrest in S phase of the cell cycle, and that this arrest was accompanied by the activation of the ATR-hChk1 checkpoint pathway (1). These results prompted speculation that escape from the ATR-hChk1 –induced growth arrest confers a proliferative advantage to malignant clones in the developing tumor. At the same time, these clones will gain an increased level of genomic instability due to the disruption of the ATR-dependent S phase checkpoint. Thus, hypoxia-induced S phase arrest may fuel BCa tumorigenesis in part by increasing the rate of gene mutation in the affected BCa cells. We further contend that errors incurred during DNA replication represent a major source of mutations that drive BCa development.

The original goal of this project was to further understand the interplay between tumor hypoxia-reoxygenation, the ATR-hChk1 checkpoint pathway, and malignant progression in human BCa. Our initial objective was to establish relevant in vitro culture systems involving human breast epithelial cells (hBrEC) that are either precancerous or fully malignant. These model systems were generated in order to dissect the roles of surveillance proteins such as ATR, hChk1 and BRCA1, as suppressors of tumor progression, and the impact of defective ATR-mediated checkpoint activation on genetic instability and the emergence of fully tumorigenic BCa clones. Finally, using the knowledge gained from these studies, planned to explore rational therapeutic strategies targeted against cells bearing defective ATR-dependent S phase checkpoints. As described below, unexpected observations made during the early stages of this project led us down an unforeseen path of investigation that has profound implications for both the etiology and therapy of BCa.

BODY

Task 1: To define the role of the ATR checkpoint pathway in hypoxia-induced cytostasis, and to determine whether hypoxic adaptation promotes genetic instability in human BCa cells

In pursuing the Task 1 studies for this project we uncovered a novel mechanism that impacts not only on our understanding of the interplay between hypoxia and genetic instability in BCa, but also on the tumor responsiveness to a major class of cancer chemotherapeutic agents. We presented these initial results in the progress reports for years 1 and 2, and during year 3, we focused on a highly related investigative path, the results of which were published in a top tier journal (*Molecular Cell*) (Appendix I, manuscript #2), and highlighted on the DOD BRCP website in 2005. The novel findings presented in this paper offered some unexpected insights into the mechanisms whereby BCa cells bypass the replication checkpoint under hypoxia and other replication stress-inducing conditions, and acquire resistance to a clinically important class of anticancer agents. Importantly, our results also revealed a rational strategy to overcome this mechanism of replication stress resistance in BCa cells. Finally, a second unexpected observation led us to discover a previously unanticipated connection between ATR and components of the translesion synthesis (TLS) machinery in mammalian cells. A manuscript describing these new findings is now in preparation. Once again, defects in this ATR-dependent genome repair mechanism are expected to contribute to an increased frequency of gene mutations in cell exposed to environmentally- or therapeutically-induced replication stress.

As outlined in our original application, we investigated the possibility that the ATR-hChk1 pathway protects cancer cells from replication stress induced by low oxygen and nutrient conditions, which are frequently observed in developing, avascular microtumors. Our rationale was that a number of tumor suppressor genes relevant to breast cancer play major roles in the recovery of stalled replication forks of replication arrest induced by various genotoxic insults, including, we believe, hypoxia-reoxygenation-induced stress. Furthermore, several of our most successful anticancer drugs, including camptothecin and gemcitabine, target S-phase, and we hypothesize that the therapeutic indices of these drugs stem in part from their selective effects on tumor cells that are struggling to complete S phase due to stressful environmental conditions. In the Year 1 progress report, I presented evidence that hypoxia sensitizes BCa cells to killing by clinically relevant concentrations of CPT. During the course of these studies, we made the unexpected observation that exposure of MCF-7 BCa cells to hypoxia caused the disappearance of the hChk1 kinase (Year 2 progress report), a key target of ATR in the replication stress-response pathway (2, 3). We also observed striking decreases in hChk1 protein levels in cells exposed to genotoxic agents, including CPT, which are known to impair replication fork progression in S phase cells (Year 2 progress report and Appendix I, manuscript #2).

Further experimentation revealed that the loss of hChk1 in human BCa, as well as normal epithelial cell lines, was due to the degradation of the protein by the ubiquitin-proteasome pathway. Using CPT as the replication stress-inducing agent, we showed that the loss of hChk1 could be largely rescued by treatment of the cells with the proteasome inhibitor, LLnV (Appendix I, manuscript #2). Remarkably, we discovered that the signal for degradation of hChk1 was the very same signal that triggered hChk1 activation in CPT- or hypoxic cells – the phosphorylation of hChk1 at Ser-345 by the ATR kinase (Appendix I, manuscript #2). A search of the literature indicated to us that phosphorylation of several proteins, including a number of cell-cycle regulatory proteins, marked these proteins for poly-ubiquitination by a family of ubiquitin E3 ligases known as the cullin-ring ligases (CRLs) (4, 5). Seven cullin subunits are expressed in human cells. Using both overexpression and small-interfering RNA (siRNA) strategies, we examined the potential roles of these cullins in CPT-induced hChk1 degradation, and two – cullin 1 and cullin 4A – emerged as key regulators of hChk1 degradation in MCF-7 and other cancer cell types. We are currently searching for the F-box proteins that associate with cullin 1 and cullin 4A to form the E3 ligases that target the phosphorylated, activated form of hChk1 for proteolysis.

How do these findings apply to BCa development and therapy? Compelling evidence indicates that Chk1 function is essential for the viability of normal cells, due to the fact that, even under optimal conditions, replication forks are prone to stalling due to base misincorporation errors or aberrantly structured DNA (e.g., fragile sites) (6, 7). Stalled replication forks must remain viable until the initiating insult is repaired, and Chk1 is required for replication fork viability and replication re-initiation after stalling (8). In the absence of Chk1, forks disassemble, and the host cell is unable to completely replicate its genome. Because the damage to the genome is typically massive under these conditions, cell death due to apoptosis or premature chromosome condensation is an inevitable consequence of replication fork breakdown. Our discovery that ATR-dependent phosphorylation of hChk1 both activates this kinase and marks it for degradation may seem counter-productive to the goal of preserving cell viability in the face of genotoxic stress. However, we hypothesize that the replication stress pathway operates continuously during a normal S-phase, and that sub-populations of hChk1 molecules are continuously activated in order to maintain the movement of individual replication forks. Under normal circumstances, this pool of activated hChk1 must be removed shortly after it performs its essential function as a countermeasure to replicative stress. Otherwise, the accumulation of activated hChk1 molecules would likely reach a critical threshold, above which otherwise undamaged cells would be unable to complete S-phase. This coupled mechanism of activation and degradation works nicely under conditions of normal-low level replicative stress, but likely leads to a pathological loss of hChk1 after prolonged or high-intensity stress to the DNA replication machinery. The hypoxic tumor microenvironment provides one such setting, and cancer chemotherapy with agents such as CPT, 5-FU, and gemcitabine represent another situation in which extreme loss of hChk1 may lead to irreversible S-phase arrest and, ultimately cell death.

The relevance of this novel mechanism of hChk1 regulation to cancer therapy was established in dramatic fashion by the finding that this mechanism was abrogated in certain drug-resistant cell lines, including the BCa line, MDA-MB-231. CPT is an effective antitumor agent, but resistance to this drug is a major problem in the clinic. Our studies demonstrate that the loss of hChk1 protein is intimately related to the cytotoxic activity of CPT, and that disruption of the Chk1 degradation pathway leads to hChk1 resistance (Year 2 progress report). The significance of this finding was documented by results indicating that knockdown of hChk1 expression with siRNA reversed the CPT resistance of MDA-MB-231 cells (Year 2 progress report). Thus, this research project has not only shed new light on the antitumor mechanism of CPT, but also reveals a common pathway leading to CPT resistance, and likely resistance to other S-phase genotoxins as well. An important question that remains to be addressed is whether actual tumors from BCa patients (particularly those with late-stage disease) display similar defects in the Chk1 degradation pathway. Our results suggest that this alteration would be correlated with a poor prognosis; however, the present findings offer a potential therapeutic strategy that could significantly improve the clinical outcome in this sub-group of BCa patients. If one were to treat such patients with a small-molecule inhibitor of ATR or Chk1, it might be possible to restore the sensitivity of these cells to conventional cytotoxic therapies. Fortunately, a number of pharmaceutical companies are actively engaged in such inhibitors, and the first early clinical trials with inhibitors of Chk1 are now underway.

Task 2: To determine whether dampening of the ATR-dependent checkpoint pathway increases human BCa tumorgenicity in immunodeficient mice

Because of the potential significance of our preliminary findings on hChk1 stability, we deferred the mouse model experiments in order to focus our resources on the Task 1 related studies. Although the principal investigator of this project has recently closed his academic lab in order to assume a new position as Vice President and Head of Oncology Discovery at Wyeth, the lead postdoctoral fellow (Dr. You-Wei Zhang) supported by this DOD-BRCP grant is now completing his training in Dr. Tony Hunter's laboratory at the Salk Institute, with funding from the Susan B. Komen Breast Cancer Foundation. He is continuing to probe the role of the ATR-Chk1 pathway in breast carcinogenesis, and the proposed task 2 studies are scheduled to be performed in Dr. Hunter's lab. It is important for the DOD to be aware of the fact that, in addition to the reportable data that stemmed from this project, the research program prompted Dr. Zhang, a highly promising young scientist, to commit his research career to studies of BCa biology and therapy

KEY RESEARCH ACCOMPLISHMENTS

- Established a panel of hBrEC cell lines in the laboratory

- Established the hardware that allows for cell culture in hypoxia and nutrient-deprived conditions
- Demonstrated that loss of ATR function impairs the recovery of BCa cells from hypoxia-induced S phase arrest
- Demonstrated that both hypoxia and S-phase specific anticancer agents activate the ATR-hChk1 pathway, leading to the poly-ubiquitination and proteolytic degradation of hChk1.
- Discovered a novel mechanism whereby the activation of ATR by replication stress leads to the phosphorylation of hChk1 at Ser-345, a modification that simultaneously activates the Chk1 kinase domain and targets the protein by cullin 1- and cullin 4A-containing E3 ligases.
- Provided novel insights into the antitumor mechanism of CPT, and demonstrated that breast cancer cells acquire resistance to CPT through disruption of the hChk1 degradation pathway.
- Generated inducible hChk1 cell lines for use in further studies.
- Provided a foundational set of findings that launched a fundamentally important new area of cancer research – the role of aberrant checkpoint protein degradation in cancer development and therapeutic responsiveness

REPORTABLE OUTCOMES

Manuscripts

- Zhang YW, Hunter T, and Abraham RT: Turning the replication checkpoint on and off. *Cell Cycle* 5(2):125-128, 2006.
- Zhang YW, Otterness DM, Chiang GG, Xie W, Liu YC, Mercurio F, and Abraham RT.: Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. *Mol. Cell.* 19(5):607-618, 2005.
- Tsao CC, Geisen C, and Abraham RT: Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. *EMBO J.* 23:4660-4669, 2004.
- Tsao CC, Geisen C, Rabindran S, and Abraham RT: Bidirectional interplay between ATR and the translesion synthesis machinery during replication stress. Manuscript in preparation, 2006.

Abstracts

- Zhang, YW and Abraham RT "Regulation and therapeutic potential of Chk1 kinase in breast cancer". Abstract presented by You-Wei Zhang at the "Era of Hope" Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, June 8-11, 2005. (See Appendix II, Abstract #1)

Presentations (selected)

- Invited Speaker for the GSA's Distinguished Lecturer Seminar Series, "CHecKing in on DNA

Replication Fidelity in Human Cells" Mayo Clinic, Rochester, MN, September 8, 2004.

- Invited Speaker, MD Anderson Cancer Center, Department of Molecular and Cellular Oncology, "DNA Damage-Induced Chk1 Degradation as a Determinant of the Response to Cancer Chemotherapy", Houston, TX, April 27, 2005.
- Invited speaker, CELLULAR RESPONSES TO DNA DAMAGE, Copenhagen, Denmark, August 21-25, 2005.

Cell lines

- The cell lines and cDNA constructs generated during this project (human BCa and inducible hChk1) are being made available to the general research community

Personnel receiving pay from research effort:

- Robert T. Abraham, Ph.D. – Principal Investigator
- You-Wei Zhang, Ph.D. – Postdoctoral Associate
- Cheng-Chung Tsao, Ph.D. – Postdoctoral Associate
- Diane M. Otterness – Senior Research Assistant
- Caroline B. Ho, M.Sc. – Senior Research Assistant

Funding applied for based on this award

Dr. You-Wei Zhang, a post-doctoral associate supported by this award, applied for and received a post-doctoral fellowship entitled "Roles of Chk1 Kinase in Breast Cancer Etiology and Therapy" from the Susan G. Komen Foundation (PDF0503489). Dr. Abraham has recently assumed a position as Vice President and Head of Oncology Discovery at Wyeth, and is no longer applying for any extramural funding.

An RO1 application entitled "Checkpoint Kinase Chk1 in Cancer Biology and Therapy" was submitted to the NIH. Dr. Tony Hunter will be the Principal Investigator and Dr. Abraham will serve as co-investigator on this project.

CONCLUSIONS

This initial DOD Breast Cancer Research Program-funded project was highly successful from both the scientific and the career development viewpoints. During this project, we generated several new human breast cancer cell lines, and developed both the technology and expertise to explore the role of hypoxia-reoxygenation in cancer cell survival, proliferation, and evolution. These studies revealed that replication stress leads to the activation of an ATR/hChk1-mediated checkpoint response that allows these cells to restart stalled replication forks after the initiating insult is resolved. Our studies also demonstrated that persistent or high-intensity stress, whether derived from hypoxia or other environmental challenges, leads to the degradation of hChk1, and leads in turn to massive genetic instability in the surviving cells. Furthermore, this hChk1 degradation response plays a key role in therapeutic sensitivity of BCa cells to a clinically significant class of anticancer drugs. Remarkably, we discovered that certain BCa cell lines display an aberrant Chk1 degradation response, which is strongly correlated with resistance to CPT and other cytotoxic agents that trigger the replication stress response. In many cases, these drug-resistant cells fail to degrade hChk1 in response to CPT exposure, which allows the cells to maintain viability and resume cell proliferation after termination of CPT therapy. We (Drs. Zhang, Hunter, and myself) will continue to explore the mechanism and consequences of hChk1 degradation in BCa and other malignant cell lines and will move our newly generated, inducible cell lines to the *in vivo* antitumor studies proposed in task 2 of the original application.

To conclude this project summary, I would like to emphasize that the research took an unexpected turn due to the observational prowess of a promising young scientist, Dr. You-Wei Zhang. As is often the case in research, the serendipitous finding sometimes far outweighs the research plan in terms of novelty and impact, largely because the research plan reflects a planned series of responses to a sequence of events that the investigator believes will occur based on the existing knowledge. The serendipitous finding often takes the investigator outside of the scope of the existing knowledge, and tests one's ability to respond appropriately when one enters relatively uncharted territory. The research performed in this proposal provided the basis for research that is now being actively pursued in many labs, and, as stated above, it provided an unanticipated insight into the mechanism whereby breast cancer cells become resistant to certain clinically important chemotherapeutic agents. Finally, and perhaps most importantly, this DOD-BRCP-funded work launched a highly promising young post-doctoral researcher into a career in breast cancer research.

REFERENCES

1. Hammond, E. M., Denko, N. C., Dorie, M. J., Abraham, R. T., and Giaccia, A. J. Hypoxia links ATR and p53 through replication arrest. Molecular and Cellular Biology, *22:* 1834-1843, 2002.
2. Abraham, R. T. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes & Development, *15:* 2177-2196, 2001.
3. Bartek, J., Lukas, C., and Lukas, J. Checking on DNA damage in S phase. Nat Rev Mol Cell Biol, *5:* 792-804, 2004.
4. Spruck, C. H. and Strohmaier, H. M. Seek and destroy: SCF ubiquitin ligases in mammalian cell cycle control.[see comment]. Cell Cycle, *1:* 250-254, 2002.
5. Deshaies, R. J. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol, *15:* 435-467, 1999.
6. Cimprich, K. A. Fragile sites: breaking up over a slowdown. Curr Biol, *13:* R231-233, 2003.
7. Casper, A. M., Nghiem, P., Arlt, M. F., and Glover, T. W. ATR regulates fragile site stability. Cell, *111:* 779-789, 2002.
8. Zachos, G., Rainey, M. D., and Gillespie, D. A. Chk1-dependent S-M checkpoint delay in vertebrate cells is linked to maintenance of viable replication structures. Mol Cell Biol, *25:* 563-574, 2005.

APPENDICES

APPENDIX I: *Manuscripts*

1. Zhang YW, Hunter T, and Abraham RT: Turning the replication checkpoint on and off. *Cell Cycle*5(2):125-128, 2006.

2. Zhang YW, Otterness DM, Chiang GG, Xie W, Liu YC, Mercurio F, and Abraham RT.: Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. *Mol. Cell.* 19(5):607-618, 2005.

3. Tsao, CC, Geisen, C, and Abraham RT: Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. *EMBO J.* 23:4660-4669, 2004.

APPENDIX II: *Abstracts*

1. Zhang, YW and Abraham RT "Regulation and therapeutic potential of Chk1 kinase in breast cancer." "Era of Hope" Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, June 8-11, 2005.

Abstract #1

Zhang, YW and Abraham RT "Regulation and therapeutic potential of Chk1 kinase in breast cancer." "Era of Hope" Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, June 8-11, 2005.

The Chk1 kinase is a major effector of S-phase checkpoint signaling during the cellular response to genotoxic stress. Here we report that replicative stress induces the poly-ubiquitination and degradation of Chk1 in human cells. This response is dependent on the phosphorylation of Chk1 at Ser-345, a known target site for the upstream regulatory kinase, ATR. The ubiquitination of Chk1 is mediated by E3 ligase complexes containing Cul1 or Cul4A. Treatment of cells with the anticancer agent, camptothecin (CPT), triggers Chk1 destruction, which blocks recovery from drug-induced S-phase arrest and leads to cell death. Defects in Chk1 downregulation are associated with resistance to the cytotoxic effect of CPT. These findings indicate that ATR-dependent phosphorylation of Chk1 delivers a signal that both activates Chk1 and ultimately marks this protein for proteolytic degradation. Furthermore, replication stress-induced Chk1 destruction may play a crucial role in tumor cell killing by CPT and related drugs

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Extra View

Turning the Replication Checkpoint On and Off

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Chk1, DNA damage, replication checkpoint, ubiquitination, Cul1, Cul4A, camptothecin

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ABSTRACT

The replication checkpoint monitors the progress of DNA replication forks during S phase, and delays the firing of later replication origins when active replication forks are stalled due to collisions with damaged or abnormally structured DNA. Key components of the replication checkpoint pathway are the apical protein kinase, ATR, and its downstream target kinase, Chk1. Defects in either ATR or Chk1 function result in loss of DNA replication fidelity and cell viability, even in the absence of extrinsic genotoxic stress. Moreover, several clinically important antitumor agents, such as the camptothecins (CPTs), exert their antitumor effects by interfering with DNA replication, and hence the therapeutic response to these drugs is intimately related to signaling through the replication checkpoint. A recent report from this laboratory adds a new facet to the regulatory mechanisms that control the function and duration of checkpoint signaling through the ATR-Chk1 pathway. The results indicate that replication stress induced by a variety of agents, including CPT and deep hypoxia, triggers the ubiquitin-dependent degradation of the checkpoint kinase Chk1 in both normal and transformed human cells. This review provides an overview of the study's major findings, together with their implications for both replication checkpoint function and tumor responsiveness to CPT and related anticancer drugs.

A paramount objective of the eukaryotic cell cycle is to generate a complete and accurate copy of the genome before the host cell undergoes mitotic cell division. Even in the absence of extrinsic genotoxic stress, the fidelity of DNA replication is threatened by cell-intrinsic events that lead to base incorporation errors, and to the stalling of active DNA replication forks. Exposure of cells to DNA-damaging agents, such as UV light, DNA polymerase inhibitors, and certain clinically important antitumor drugs (e.g., CPT or gemcitabine), greatly increases the risk of erroneous or incomplete copying of the genome during S phase. The replication checkpoint plays central roles in monitoring both replication fidelity and replication fork progression through the parental DNA template in eukaryotic cells. Recent findings suggest that components of the DNA replication machinery itself actually serve as sensors of damaged or abnormally structured template DNA in the path of active replication forks.[1,2] Downstream of these sensor proteins resides a more specialized group of checkpoint signaling proteins that includes (in mammalian cells) the checkpoint kinases, ATR and Chk1, which phosphorylate a host of regulatory and effector proteins that block the firing of additional DNA replication origins, repair damaged DNA, and promote the stabilization and restart of stalled replication forks after the initiating insult is removed.[3,4]

Given that DNA synthesis places heavy demands on cell metabolism and bioenergetics, it is not surprising that that this process is sensitive to environmental parameters, including the availability of oxygen and nutrients, which are delivered to mammalian tissues via the microvasculature. Accumulating evidence supports the idea that vascular insufficiency leads to chronic replication stress in early-stage tumors. The harsh tumor microenvironment eventually selects for malignant cells bearing mutations that impair or override the replication checkpoint. The selected tumor cells are poised to acquire additional alterations in S-phase and/or mitotic checkpoints that lead to high-level genetic instability and, in turn, an increased rate of malignant progression.[4,5] These recent findings underscore the importance of the replication checkpoint in both normal cell physiology, and in the pathological events leading to the evolution of high-grade tumors in humans. The replication checkpoint is relevant not only to cancer development, but to cancer therapy as well. Several widely used anticancer drugs, such as CPT and gemcitabine, are classified as S-phase-specific genotoxic agents. In the therapeutic setting, the replication checkpoint may play a key role in protecting both normal and malignant cells from the cytotoxic effects of these agents.

In response to replication stress, ATR is activated by mechanisms involving the binding of the protein kinase, together with its non-catalytic ATRIP subunit, to replication protein A (RPA)-coated, single-stranded DNA. The activated ATR kinase, in conjunction with a number of additional checkpoint proteins, including claspin and the Rad checkpoint complex, recruits the Chk1 kinase to the site of DNA damage, and phosphorylates Chk1 at two serine-glutamine motifs (Ser-317 and Ser-345) in its carboxyl-terminal domain. Through a mechanism that is poorly understood, these phosphorylation events provoke the activation of the amino-terminal kinase domain of Chk1. Known, but certainly not the only substrates for Chk1 are the protein tyrosine phosphatases, Cdc25A and Cdc25C, which play central roles in the G_1-S and G_2-M transitions, respectively.[4] Phosphorylation of these Cdc25 proteins by Chk1 marks them for ubiquitination and proteolytic degradation, thereby blocking cell cycle progression into S and M phases during periods of replication stress. This checkpoint mechanism is commonly referred to as the 'S-phase checkpoint'. Our understanding of the mechanisms whereby Chk1 activation preserves the viability of stalled replication forks and delays the firing of later replication origins (responses commonly associated with 'replication checkpoint' function) is far less detailed, although steady progress is being made in this area.[6,7]

Previous results from Amato Giaccia's laboratory had established that deep hypoxia results in a DNA replication block in mammalian cells, and an accompanying activation of the ATR-Chk1 checkpoint-signaling pathway.[8,9] Based on these results, we postulated that, in developing tumors, hypoxic cancer cells might be heavily dependent on the replication checkpoint for preservation of cell viability, and for resumption of cell-cycle progression after reoxygenation of the tumor tissue. An extension of this hypothesis was that antitumor agents, such as CPT, which place additional stress on the replication checkpoint, might owe their therapeutic effects in part to the selective killing of cancer cells coping with chronic replication stress due to oxygen and nutrient deprivation. Although this hypothesis still merits serious consideration, the ensuing studies led us to pursue a more molecular path that provided some more generalizable insights into the mechanisms whereby both normal and transformed cells control the intensity and duration of ATR-Chk1 signaling during S phase.

In the initial experiments, we noted that exposure of several cancer cell lines to deep hypoxia ($O_2 < 0.1\%$) for moderate time periods (6–16 h) resulted in a strking reduction in the level of the Chk1 protein. Similar results were obtained when cells were treated for 4–8 h with the topoisomerase I inhibitor, CPT, under normoxic conditions, suggesting that this response was generally triggered by conditions that cause chronic replication stress. Indeed, other known S-phase-targeted genotoxic agents, such as methylmethanesulfonate and aphidicolin, also provoked a significant downregulation of the Chk1 protein in various human cell lines.[10] The decrease in steady-state levels of Chk1 protein observed in these experiments could reflect either stress-induced transcriptional repression of the *CHK1* gene, or post-transcriptional alterations leading to increased turnover of the Chk1 polypeptide. In reality, the ensuing experiments indicated that both transcriptional and post-transcriptional mechanisms contributed to the reduction in Chk1 protein level induced by high-intensity replication stress. In p53-positive A549 lung carcinoma cells, CPT exposure caused a moderate (~30%) decrease in the steady-state level of Chk1 mRNA, and a considerably more dramatic reduction (~70%) in the level of Chk1 protein (ref. 10 and unpublished results). These results were consistent with an earlier report, which indicated that p53 functions as a transcriptional repressor of the *CHK1* gene.[11,12] However, studies with both p53-positive and -negative cell lines demonstrated that decreases in *CHK1* gene transcription were not the major driver for the loss of Chk1 protein seen in CPT-treated cells. As we suspected, pulse-chase experiments revealed that CPT exposure not only provoked a strong increase in Chk1 activity (monitored by phosphorylation of the protein at the ATR-dependent Ser-317 and Ser-345 residues), but also caused a ~2-fold decrease in half-life of the Chk1 polypeptide. The decrease in Chk1 protein was substantially suppressed by simultaneous exposure of the CPT-treated cells to a proteasome inhibitor (e.g., LLnV), indicating that chronic replication stress imposed by CPT and other S-phase genotoxins leads to a dramatic increase in the rate of proteasome-mediated Chk1 degradation. Furthermore, treatment of the cells with LLnV alone provoked an increase in basal Chk1 expression levels, suggesting that proteasomal degradation of Chk1 occurred during normal cell cycle progression. The latter findings are consistent with the idea that the ATR-Chk1 surveillance pathway is tonically active in S-phase cells, in the absence of exogenously added genotoxic agents.

A more surprising finding was that the sequence of events leading to Chk1 activation exhibited partial overlap with those that targeted this protein kinase for proteasomal degradation. The results of pharmacological and RNA interference experiments highlighted a central role for ATR in the downregulation of Chk1 during chronic replication stress. Although ATR is known to phosphorylate Chk1 at two sites, Ser-345 and Ser-317, during replication checkpoint activation, the phosphorylation of Ser-345 was found to be particularly important for the induced destabilization of Chk1. Ser-345 phosphorylation was required for CPT-induced Chk1 ubiquitination, a common prelude to proteasome-mediated protein destruction. These findings add Chk1 to the growing list of protein kinaeses that are targeted for ubiquitination by phosphorylation at specific sites[13,14] Indeed, the most well-defined Chk1 substrate, Cdc25A, is marked for ubiquitination and degradation following phosphorylation by activated Chk1.[15,16] In this case, phosphorylation of Cdc25A by Chk1 drives the recruitment of an SCF (Skp1-Cullin-F box protein)-type E3 ligase to Cdc25A, which mediates the poly-ubiquitination of Chk1.

Human cells express 7 cullin isoforms, and a much larger number (~68) of F-box proteins, which serve as the substrate-binding subunits of the SCF E3 ligases.[17,18] The F-box protein that binds to phosphorylated Cdc25A is β-TrCP,[15,16] which is also a component of the SCF complex that mediates ubiquitination of the IκB subunit of NF-κB.[13] The sequence of events leading to proteasomal degradation of Chk1 show some interesting parallels with those responsible for the destruction of Cdc25A during activation of the S-phase or replication checkpoint. Our findings suggest that replication stress leads to the phosphorylation of Chk1 at Ser-345, which simultaneously activates Chk1 and targets this protein kinase for ubiquitination by two SCF-type E3 ligases (Fig. 1). The conclusion that two E3 ligases mediate Chk1 ubiquitination is supported by coimmunoprecipitation, overexpression, and RNA interference experiments, which consistently identified two cullin isoforms, Cul1 and Cul4A, as proteins that physically associated with and were functionally involved in the ubiquitination of Ser-345-phosphorylated Chk1. These findings strongly suggest that two SCF-type E3 ligases, one containing Cul1, and the other, Cul4A, are recruited to Chk1 after phosphorylation of this protein kinase by ATR. How these two cullin-containing E3 ligases recognize the phosphorylated form of Chk1 remains an open question, which can only be addressed in detail after identification of the cognate F-box protein in each SCF complex.

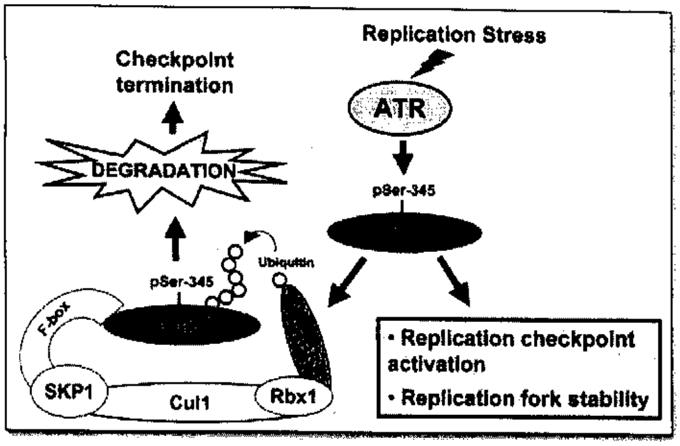

Figure 1. A coupled mechanism of Chk1 activation and degradation induced by replication stress. Replication stress leads to the ATR-dependent phosphorylation of Chk1 at Ser-345, leading to activation of this protein kinase and activation of the replication checkpoint. Ser-345 phosphorylation simultaneously marks Chk1 for ubiquitination by Cul1- or Cul4A-containing E3 ligases, and ultimately targets Chk1 for degradation by the proteasome. According to our working model, destruction of the active pool of Chk1 is required for replication checkpoint termination at individual replication forks, thereby preventing excessive accumulation of Chk1 during normal S-phase progression. During high-intensity replication stress, such as that induced by CPT, the Chk1 protein level declines to a point that is insufficient to support replication fork viability and recovery after removal of the insult, leading to irreversible S-phase arrest and cell death.

These studies indicate that the phosphorylation of Chk1 by ATR activates the kinase, and concomitantly sets in motion a series of events that leads to the destruction of the activated Chk1 (Fig. 1). As stated above, Chk1 function is essential for high-fidelity DNA replication and cell viability during normal S-phase progression. It may seem counterintuitive to propose that the mechanism that activates Chk1 would also mark this critically important protein kinase for destruction by the proteasome. However, a more detailed analysis of the relative time courses of Chk1 activation and ubiquitination revealed that, whereas Chk1 phosphorylation (and by inference Chk1 activity) increased within minutes of CPT exposure in S-phase cells, Chk1 downregulation was not readily detected until 2–4 h after drug treatment (unpublished results).[10] The delayed degradation of Chk1 presumably allows sufficient time for the activated protein kinase to exert its protective effects at stalled replication forks prior to its modification by SCF E3 ligases. We hypothesize that the timely destruction of Chk1 allows for the termination of replication checkpoint signaling, thereby allowing active replication forks to resume their DNA-copying activity, and permitting the firing of later origins of replication. During a normal S phase, we envision that a relatively small population of Chk1 molecules is continuously active as replication forks encounter local impediments (e.g., fragile sites or damaged bases) to fork progression. The coupled degradation of these active Chk1 molecules insures that these checkpoint mediators do not accumulate to the point where S-phase progression is inappropriately delayed or even blocked during the normal cell cycle. In the presence of considerably higher levels of replication stress, such as that imposed by exposure to CPT, far larger numbers of Chk1 molecules are activated and subsequently degraded, resulting in a decrease in steady-state Chk1 levels. In this setting, the strategy of coupled Chk1 activation and degradation backfires on the cell, because the resulting 50–80% decline in Chk1 levels leaves insufficient Chk1 to maintain replication fork viability and the reinitiation of fork progression.[10] The cell's fate is almost inevitably death due to irreversible S-phase arrest or to a catastrophic attempt to execute mitosis.

Further insights into the sequence of events initiated by ATR-dependent Chk1 phosphorylation came from subcellular fractionation experiments. In unperturbed cells, Chk1 is found primarily in the nucleus, specifically in the chromatin-enriched subcompartment. Cellular treatment with CPT triggered the phosphorylation of Chk1 (at Ser-317 and Ser-345) and the prompt release of this protein kinase from chromatin. As a soluble nuclear protein, the activated Chk1 associates preferentially with the Cul1-containing E3 ligase, which is also abundantly expressed in the nucleoplasm, and Chk1 ubiquitination proceeds in the nuclear compartment. Phosphorylated Chk1 that escapes the nucleus and enters the cytoplasmic compartment encounters mainly the Cul4A-containing E3 ligase, and also undergoes polyubiquitination. In contrast to the dynamic changes in Chk1 localization triggered by CPT exposure, the upstream kinase, ATR, remained chromatin-bound in both the absence and presence of replication stress. Why evolution has built in this two-step mechanism to insure that active Chk1 is efficiently ubiquitinated after its release from chromatin remains unclear. Based on the increasing evidence that ubiquitin modifications regulate protein kinase function as well as stability,[13] it is tempting to speculate that the Cul1-dependent ubiquitination that occurs in the nucleus is mechanistically related to a switch of activated Chk1 from a chromatin-bound to a diffusible mediator of replication checkpoint signaling. Nonetheless, our findings suggest that the temporal delay between Chk1 activation and Chk1 destruction is determined, at least in part, by the fact that the active protein kinase becomes susceptible to degradation only after its release from chromatin. Further studies are clearly needed to understand the relative roles of the nuclear Cul1-Chk1 and cytoplasmic Cul4A-Chk1 complexes in Chk1 function and turnover during S phase.

These findings have some important implications for both replication checkpoint function and for the response to certain types of cancer chemotherapeutic agents. As stated above, we speculate that the delayed degradation of activated Chk1 limits the duration of replication checkpoint signaling during low-intensity replication stress, and prevents the active protein kinase from accumulating to levels that would be deleterious to normal S-phase progression. In contrast, high-intensity stress, such as that induced by CPT and other clinically-relevant, S-phase-selective genotoxic agents (e.g., gemcitabine and cisplatin), leads to pathologic downregulation of Chk1, and, in turn, irreversible damage to stalled replication forks, culminating in cell death. The targeting of an activated protein kinase for ubiquitination and proteasomal degradation is certainly not unique to Chk1. A number of other signaling kinases, including PKC,[19] c-Abl,[20] and Wee1,[21] are also marked for ubiquitination via the same mechanism that leads to their activation. As opposed to phosphorylation-dephosphorylation, the ubiquitin-proteasome pathway represents an irreversible mechanism for protein kinase inactivation. This alternative mechanism of signal termination is particularly useful when the stimulus for the activation of the target kinase is of low intensity and chronic in nature. In this setting, proteolytic downregulation of the protein kinase provides a definitive strategy for the termination of signal output through the population of activated protein kinase molecules.

The finding that therapeutically relevant concentrations of CPT provoke a functionally significant decrease in Chk1 protein levels sheds new light on the antitumor mechanism of this drug. The most widely accepted model for cancer cell killing by CPT posits that the collision of moving replication forks with DNA-bound CPT-topoisomerase I complexes leads to lethal DNA double-strand breaks.[22] However, even in the absence of overt DNA strand breakage, these CPT-topoisomerase I-DNA complexes present roadblocks to the progression of replication forks, and hence serve as powerful stimuli for activation of the ATR-Chk1 pathway. In cell culture, "chronic" (≥ 4 h) exposure to CPT, a time course readily achieved during cancer therapy in humans, causes the downregulation of Chk1 to levels insufficient to support replication fork recovery after removal of the drug. We propose that cell death results either from irreversible S-phase arrest or entry into mitosis prior to the completion of DNA replication. The clinically-acceptable safety margin associated with CPT in cancer patients may stem from the concept that cancer cells are driven to proliferate under moderate to high-level replication stress, due, for example, to an inadequate supply of oxygen and nutrients. Therapeutic concentrations of CPT may push these stressed cells over the brink to replication fork complex collapse, whereas normal cells may have sufficient reserve in the ATR-Chk1 pathway to recover from the CPT-induced block to fork progression. The validity of this model is supported by our recent finding that several cancer cell lines that display high-level resistance to CPT exhibit clear defects in CPT-induced Chk1 degradation that cannot be attributed to mutations in the CPT target protein, topoisomerase I (unpublished results). Forced downregulation of Chk1 causes a dramatic increase in CPT sensitivity in these cells. If these in vitro findings can be extrapolated to the in vivo setting, then pharmacologic inhibition of Chk1 activity could overcome CPT resistance in the oncology clinic—a major problem that limits the efficacy of this drug. Indeed, preclinical data already supports the idea that geldanamycin (a heat-shock protein-90 inhibitor) sensitizes tumor cells to CPT by decreasing the steady-state levels of Chk1.[23] It seems obvious that further understanding of the molecular events that turn the replication checkpoint on and off will attract considerable interest from both cancer biologists and clinical oncologists, and may well lead to combination therapies that increase the clinical activities of CPT and other S phase-targeted genotoxic agents.

References

1. Cortez D, Glick G, Elledge SJ. Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. Proc Natl Acad Sci USA 2004; 101:10078-83.
2. Tsao CC, Geisen C, Abraham RT. Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. EMBO J 2004; 23:4660-9.
3. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 2001; 15:2177-96.
4. Bartek J, Lukas C, Lukas J. Checking on DNA damage in S phase. Nat Rev Mol Cell Biol 2004; 5:792-804.
5. Sherr CJ. Principles of tumor suppression. Cell 2004; 116:235-46.
6. Feijoo C, Hall-Jackson C, Wu R, Jenkins D, Leitch J, Gilbert DM, Smythe C. Activation of mammalian Chk1 during DNA replication arrest: A role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. J Cell Biol 2001; 154:913-23.
7. Shechter D, Costanzo V, Gautier J. ATR and ATM regulate the timing of DNA replication origin firing. Nat Cell Biol 2004; 6:648-55.
8. Hammond EM, Denko NC, Dorie MJ, Abraham RT, Giaccia AJ. Hypoxia links ATR and p53 through replication arrest. Mol Cell Biol 2002; 22:1834-43.
9. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J Biol Chem 2003; 278:12207-13.
10. Zhang YW, Otterness DM, Chiang GG, Xie W, Liu YC, Mercurio F, Abraham RT. Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. Mol Cell 2005; 19:607-18.
11. Gottifredi V, Karni-Schmidt O, Shieh SS, Prives C. p53 downregulates CHK1 through p21 and the retinoblastoma protein. Mol Cell Biol 2001; 21:1066-76.
12. Cummings M, Siitonen T, Higginbottom K, Newland AC, Allen PD. p53-mediated dowregulation of Chk1 abrogates the DNA damage-induced G_2M checkpoint in K562 cells, resulting in increased apoptosis. Br J Haematol 2002; 116:421-8.
13. Cardozo T, Pagano M. The SCF ubiquitin ligase: Insights into a molecular machine. Nat Rev Mol Cell Biol 2004; 5:739-51.
14. Reed SI. Ratchets and clocks: The cell cycle, ubiquitylation and protein turnover. Nat Rev Mol Cell Biol 2003; 4:855-64.
15. Jin J, Shirogane T, Xu L, Nalepa G, Qin J, Elledge SJ, Harper JW. SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. Genes Dev 2003; 17:3062-74.
16. Busino L, Donzelli M, Chiesa M, Guardavaccaro D, Ganoth D, Dorrello NV, Hershko A, Pagano M, Draetta GF. Degradation of Cdc25A by β-TrCP during S phase and in response to DNA damage. Nature 2003; 426:87-91.
17. Jin J, Cardozo T, Lovering RC, Elledge SJ, Pagano M, Harper JW. Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev 2004; 18:2573-80.
18. Kipreos ET, Lander LE, Wing JP, He WW, Hedgecock EM. cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family. Cell 1996; 85:829-39.
19. Nishizuka Y. The protein kinase C family and lipid mediators for transmembrane signaling and cell regulation. Alcohol Clin Exp Res 2001; 25:3S-7S.
20. Echarri A, Pendergast AM. Activated c-Abl is degraded by the ubiquitin-dependent proteasome pathway. Curr Biol 2001; 11:1759-65.
21. Watanabe N, Arai H, Nishihara Y, Taniguchi M, Hunter T, Osada H. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFβ-TrCP. Proc Natl Acad Sci USA 2004; 101:4419-24.
22. Pommier Y, Redon C, Rao VA, Seiler JA, Sordet O, Takemura H, Antony S, Meng L, Liao Z, Kohlhagen G, Zhang H, Kohn KW. Repair of and checkpoint response to topoisomerase I-mediated DNA damage. Mutat Res 2003; 532:173-203.
23. Flatten K, Dai NT, Vroman BT, Loegering D, Erlichman C, Karnitz LM, Kaufmann SH. The role of checkpoint kinase 1 in sensitivity to topoisomerase I poisons. J Biol Chem 2005; 280:14349-55.

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Genotoxic Stress Targets Human Chk1 for Degradation by the Ubiquitin-Proteasome Pathway

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Summary

The Chk1 kinase is a major effector of S phase checkpoint signaling during the cellular response to genotoxic stress. Here, we report that replicative stress induces the polyubiquitination and degradation of Chk1 in human cells. This response is triggered by phosphorylation of Chk1 at Ser-345, a known target site for the upstream activating kinase ATR. The ubiquitination of Chk1 is mediated by E3 ligase complexes containing Cul1 or Cul4A. Treatment of cells with the anticancer agent camptothecin (CPT) triggers Chk1 destruction, which blocks recovery from drug-induced S phase arrest and leads to cell death. These findings indicate that ATR-dependent phosphorylation of Chk1 delivers a signal that both activates Chk1 and marks this protein for proteolytic degradation. Proteolysis of activated Chk1 may promote checkpoint termination under normal conditions, and may play an important role in the cytotoxic effects of CPT and related anticancer drugs.

Introduction

DNA replication is an error-prone process that requires continuous surveillance to insure that the genome is copied accurately and completely before the cell enters mitosis. Replicative stress activates a checkpoint pathway that includes the phosphatidylinositol 3-kinase-related kinase ATR, and its downstream substrate, the Chk1 kinase (Abraham, 2001; Shiloh, 2003). The ATR-Chk1 pathway is activated by DNA lesions that impair replication fork progression, and employs components of the replication fork itself as DNA damage sensors (Bartek et al., 2004). ATR activates Chk1 by phosphorylating this protein on at least two residues (Ser-317 and Ser-345) located in a Ser/Thr-Gln-rich domain (Bartek et al., 2004).

In response to genotoxic stress, Chk1 delays cell cycle progression through S or G2 phases by inhibiting the Cdc25A and Cdc25C phosphatases (Bartek et al., 2004). Studies in Chk1-deficient cells indicate that Chk1, like ATR, is essential for normal cell proliferation (Lam et al., 2004; Liu et al., 2000; Takai et al., 2000). A recent report demonstrated that the ATR-Chk1 pathway controls the timing of early versus late replication origin firing during normal S phase (Shechter et al., 2004). In addition, this pathway maintains DNA replication through structurally unfavorable stretches of DNA, termed fragile sites in mammalian cells (Casper et al., 2002; Cha and Kleckner, 2002). The importance of Chk1 for DNA replication is highlighted by the recent report that a partial reduction in Chk1 expression causes spontaneous S phase damage in developing mammary epithelium in vivo (Lam et al., 2004). Finally, the ATR-Chk1 pathway is vitally important for the recovery of cells from replication arrest induced by genotoxic agents, such as ultraviolet (UV) light or inhibitors of DNA polymerases (Feijoo et al., 2001; Zachos et al., 2003).

A number of clinically useful anticancer agents specifically interfere with components of the DNA replication machinery. Among the most intriguing of these S phase-specific cytotoxic drugs are the camptothecins (CPTs), which function as topoisomerase I (Top1) poisons. Although the cytotoxic mechanism of CPT is only partially understood, the most widely accepted model posits that collision of moving replication forks with DNA-bound CPT-Top1 complexes generates lethal DNA double-strand breaks (Hsiang et al., 1989). CPT exposure activates the ATR-Chk1 pathway, which protects cells from Top1 poison-induced cell killing (Cliby et al., 2002; Siu et al., 2004).

During studies of Chk1 activation by agents that induce replicative stress, we observed a time-dependent downregulation of the Chk1 protein in both normal and transformed human cell lines. Further exploration of this phenomenon revealed that ATR-mediated Chk1 activation simultaneously targets this protein kinase for polyubiquitination and proteasomal degradation. Our findings suggest that replication stress-induced Chk1 degradation normally functions to limit the duration of Chk1 signaling during low-intensity replication stress, and that this response plays an important role in cancer cell killing by CPT.

Results

Genotoxic Stress Induces Chk1 Downregulation

Initial studies unexpectedly revealed that treatment of human cancer cell lines with CPT led to a marked reduction in Chk1 protein after 4–8 hr of drug exposure (our unpublished results). In subsequent experiments, we examined the effects of a broader range of genotoxic agents on Chk1 expression levels. Chk1 expression varies during the cell cycle, with peak levels of Chk1 observed during S to M phase (Kaneko et al., 1999). To avoid complications related to the effects of stress-inducing agents on cell cycle progression, we synchronized A549 lung carcinoma cells at the G_1-S

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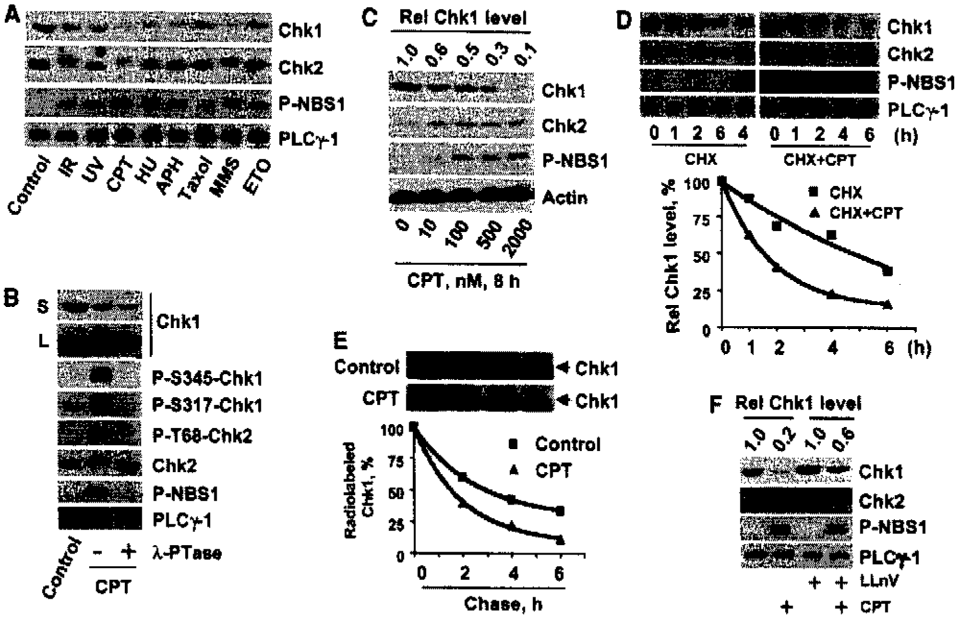

Figure 1. Proteasome-Dependent Degradation of Chk1 by Genotoxic Agents

(A) A549 cells were synchronized with double-thymidine block, and then released into S phase. One hour after release, cells were treated for 5 hr with 10 Gy IR, 100 J/m^2 UV, 500 nM CPT, 5 mM HU, 3 μg/ml APH, 1 μM taxol, 0.05% MMS, or 500 nM ETO. Cell extracts were prepared and immunoblotted with the indicated antibodies.

(B) Asynchronous A549 cells were treated for 8 hr with 500 nM CPT. Cell extracts were treated for 1 hr with buffer only or with λ-phosphatase (PTase), and then blotted with the indicated antibodies. Short (S) and long (L) exposures of the α-Chk1 immunoblot are shown. The star indicates a modified form(s) of Chk1 that is eliminated by PTase treatment.

(C) Asynchronous MCF-7 cells were treated with the indicated concentrations of CPT for 8 hr, and cell extracts were immunoblotted with the indicated antibodies. Numbers at the top of each sample lane represent the relative Chk1 protein level normalized to the control.

(D) Asynchronous A549 cells were pretreated for 15 min with 100 μg/ml cycloheximide (CHX), and then 500 nM CPT was added into the medium and cultured for the indicated times. Lower panel: Chk1 protein was determined by densitometry and normalized to the 0 hr control, which was set at 100%.

(E) HEK 293T cells were transfected with HA-Chk1 plasmid. After 48 hr, the cells were metabolically labeled for 2 hr with ^{35}S-methionine/cysteine, and chased in the presence or absence of 500 nM CPT for the indicated times. Overexpressed Chk1 proteins were immunoprecipitated with α-HA antibodies, and separated by SDS-PAGE. The relative amount of ^{35}S-labeled Chk1 protein was determined by densitometry and normalized to the 0 hr control, which was set at 100%.

(F) A549 cells were treated for 8 hr with 500 nM CPT, in the absence or presence of 4 μM LLnV during the final 4 hr in culture. Cell extracts were immunoblotted with the indicated antibodies. Numbers at the top of each sample lane represent the relative Chk1 protein level, normalized to that obtained in non-CPT-treated samples.

boundary with a double-thymidine block, and then released the cells into S phase for 1 hr prior to genotoxin exposure. Treatment of the cells with CPT, hydroxyurea (HU), methylmethanesulfonate (MMS), or aphidicolin (APH) strongly reduced Chk1 protein levels, while UV-B light and infrared (IR) (Figure 1A) caused more moderate decreases in Chk1. Higher doses of UV-B (200–400 J/m^2) provoked a more dramatic loss of Chk1 under these experimental conditions (see Figure S1A in the Supplemental Data available with this article online). In contrast, etoposide (ETO, a Top2 poison), or taxol (a microtubule inhibitor) had little effect on Chk1 expression under these experimental conditions (Figure 1A and results not shown).

A trivial explanation for the changes in expression of α-Chk1 antibody-reactive protein observed in Figure 1A is that phosphorylation of Chk1 by ATR or other kinases causes a decrease in Chk1 recognition by the α-Chk1 antibody. To address this issue, we treated cell extracts with λ-phosphatase (PTase) prior to SDS-PAGE and immunoblotting. PTase treatment effectively dephosphorylated proteins in the cell extract, as indicated by the loss of reactivity with α-phospho-Chk1 antibodies (Ser-317 and Ser-345 of Chk1) and α-phospho-NBS1 antibodies (Figure 1B). However, PTase treatment failed to reverse the decrease in α-Chk1-immunoreactive protein observed in extracts from CPT-treated cells, confirming that the drug treatment provoked an actual decrease in the steady-state levels of Chk1 protein. In contrast, the apparent decrease in Chk2 expression in CPT-treated cell extracts was reversed by PTase treatment, indicating that phosphorylation of this protein significantly interfered with its recognition by α-Chk2 antibodies.

The reduction in Chk1 protein induced by CPT was evident at drug concentrations as low as 10 nM (Figure 1C), which are well within the range of blood concentrations achieved in cancer patients receiving this drug (Rivory et al., 1997). The loss of Chk1 was also time dependent, beginning after 2 hr of cellular exposure to CPT (Figure S1B).

Posttranscriptional Effects of Genotoxic Stress on Chk1 Expression

To determine whether CPT exposure alters Chk1 protein stability, we treated A549 cells with cycloheximide

(CHX) to block protein synthesis, and then monitored the rates of decline of the endogenous Chk1 polypeptide in control versus CPT-treated cells by immunoblotting. The results indicated that the CPT exposure decreased the apparent half-life of the Chk1 protein from approximately 4.8 hr to 1.7 hr (Figure 1D). To confirm this observation, we transfected HEK 293T cells with a wild-type HA-Chk1 expression plasmid, and monitored the half-life of the HA-tagged protein by pulse-chase analysis in ^{35}S-methionine/cysteine-labeled cells. Exposure of the cells to CPT decreased the half-life of the recombinant Chk1 protein from 3.4 hr in control HEK 293T cells to 1.7 hr (Figure 1E). This transfection experiment also effectively rules out any potential contribution of genotoxin-induced Chk1 promoter repression (Gottifredi et al., 2001) to the observed downregulation of Chk1 protein.

Chk1 Downregulation Is Proteasome Dependent

To determine whether proteasomal activity was involved in Chk1 downregulation, we treated asynchronous A549 lung carcinoma cells with CPT (Figure 1F), MMS, or IR (Figure S2A) in the absence or presence of the proteasome inhibitor LLnV. In each case, LLnV treatment attenuated the decrease in Chk1 expression induced by these genotoxic agents. Similar results were obtained in experiments with a second proteasome inhibitor, MG-132 (Figure S2B). Furthermore, proteasome-dependent degradation of Chk1 was observed in other transformed cell lines (U2-OS, MCF-7, and HEK 293T), as well as nontransformed, human diploid BJ fibroblasts (Figures S2C and S2D). Interestingly, treatment of these cells with LLnV alone often caused a noticeable increase in the basal level of Chk1 protein, suggesting that Chk1 is subjected to proteasomal degradation during the normal cell cycle (Figure 1F).

Chk1 Is Required for Resumption of S Phase Progression after Genotoxic Stress

Chk1 is centrally involved in both the induction of and recovery from S phase checkpoint activation in cells exposed to replicative stress (Feijoo et al., 2001; Zachos et al., 2003). Treatment of either MCF-7 or A549 cells with CPT under conditions that provoked little or no Chk1 downregulation (i.e., 100 nM drug; 4 hr exposure time) resulted in cell cycle progression through S phase and accumulation of the cells in G_2-M phase during subsequent culture in drug-free medium. However, protracted replication stress (500 nM CPT; 8 hr exposure time) caused a loss of Chk1, accompanied by an S phase arrest that was partially reversed by suppression of Chk1 degradation with LLnV (data not shown).

Qualitatively similar results were obtained when Chk1 activity was suppressed with the pharmacologic inhibitor UCN-01 (Busby et al., 2000). In these studies, the cells were exposed to genotoxic agents under lower intensity stress conditions that did not trigger significant loss of Chk1 (Figure S3A). In the absence of UCN-01, the Chk1-proficient cells recovered from CPT-, MMS-, or HU-induced replication stress, accumulating in G_2-M phase after removal of the genotoxic agents. Addition of UCN-01 during the recovery period stalled S phase progression in these samples. In contrast, cells treated with taxol, an agent that does not interfere with DNA replication, accumulated in G_2-M phase in the absence or presence of UCN-01. Thus, UCN-01 specifically inhibits DNA replication in cells that have been exposed to S phase-targeted genotoxic agents.

In subsequent studies, we synchronized A549 cells at the G_1-S boundary with a double-thymidine block, and then released the cells into S phase, in medium containing 500 nM CPT alone, or CPT plus 2 μM LLnV. In the absence of LLnV, Chk1 was downregulated in the CPT-treated cells, and approximately 60% of these cells remained arrested in S phase after 16 hr in culture. Treatment of the cells with the proteasome inhibitor attenuated the loss of Chk1 protein induced by CPT, and concomitantly reduced the percentage of S phase-arrested cells from 60% to 40% (Figure 2A). Similar results were obtained with exponentially growing A549 and MCF-7 cells (data not shown). LLnV treatment also reduced the number of hypodiploid cells in the CPT-treated population by 50% (Figure 2A, middle panel). Conversely, inhibition of the residual Chk1 activity in CPT-treated cells with UCN-01 abolished recovery from the CPT-induced S phase arrest, and dramatically increased the proportion of hypodiploid cells. Similar results were obtained with A549 cells in which Chk1 levels were reduced by transfection with Chk1-specific siRNA (Figure S3B). The correlation between Chk1 downregulation and replication arrest was confirmed by immunofluorescence microscopy, which showed that Chk1-depleted cells failed to resume DNA synthesis, as measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation after removal of CPT from the culture medium (Figure 3). Collectively, these findings suggest that the proteasome-mediated downregulation of Chk1 is causally related to the S phase arrest and cell death induced by CPT exposure.

To further explore this hypothesis, we generated a stable MCF-7 subline that expressed a conditionally active form of Chk1. The full-length, FLAG-tagged Chk1 protein was fused with a mutated estrogen receptor (ER) ligand binding domain that selectively binds to the synthetic estrogen analog 4-hydroxytamoxifen (4-OHT) (Littlewood et al., 1995). The level of FLAG-Chk1-ER expression in these stable transfectants was approximately 2-fold higher than that of the endogenous Chk1 protein in MCF-7 cells (our unpublished results). In the absence of 4-OHT, the FLAG-Chk1-ER fusion protein is improperly folded, relatively unstable, and is not recognized as a substrate for ATR in IR- or CPT-treated cells (Figure 2B, right panel and our unpublished results). Treatment of the host cells with 4-OHT generates a biologically active FLAG-Chk1-ER protein, as indicated by genotoxin-induced Ser-345 phosphorylation (Figure 2B, right panel), and by α-FLAG immune complex kinase assays with GST-Cdc25C as the substrate (results not shown).

To determine whether conditional overexpression of FLAG-Chk1-ER suppressed CPT-induced S phase arrest, the transfected MCF-7 cells were synchronized at G_1-S boundary with a double-thymidine block, and released into S phase in the absence or presence of 4-OHT. As observed previously, exposure of the S phase cells to CPT caused a clear reduction in the expression of the endogenous Chk1 protein (Figure 2B, right panel). Notably, CPT exposure also reduced the level of FLAG-Chk1-ER fusion protein in the 4-OHT-treated cells, but

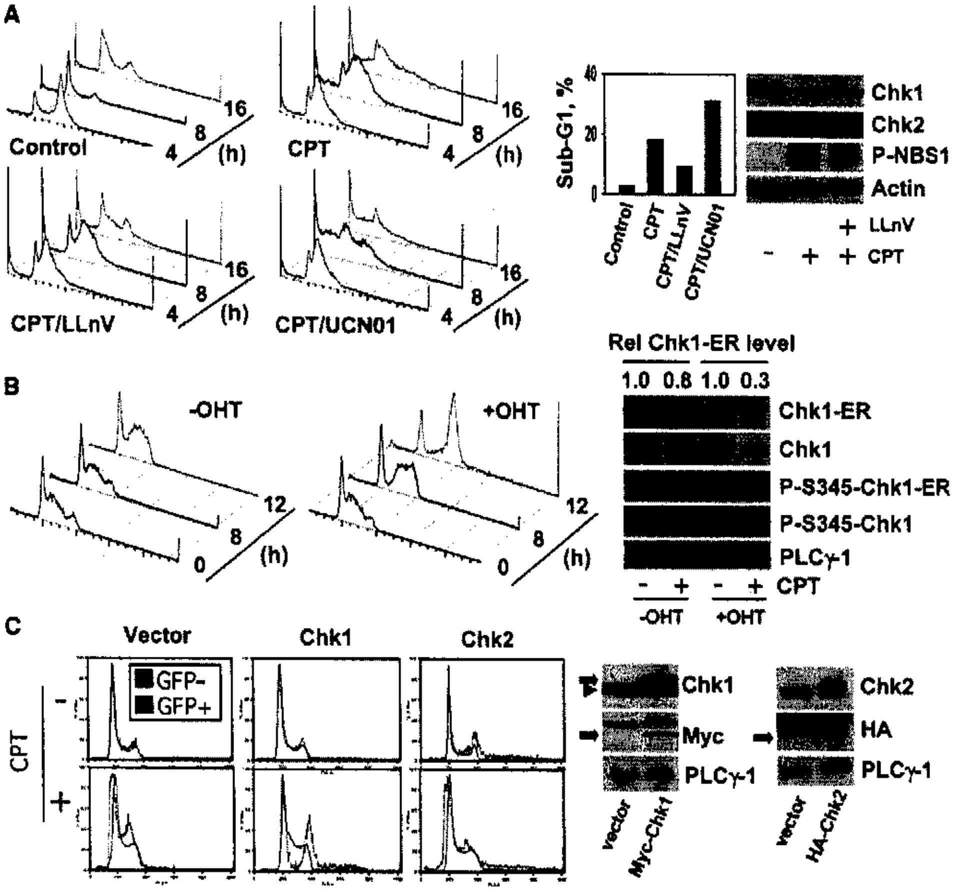

Figure 2. Role of Chk1 in Cellular Recovery from Replicative Stress

(A) A549 cells were synchronized with a double-thymidine block. The cells were released into S phase in the presence of 500 nM CPT, with or without 2 μM LLnV. After 4 hr, the cells were cultured for the indicated times in drug-free medium, in the absence or presence of 135 nM UCN-01. Cell cycle distributions were analyzed by flow cytometry. Percentages of cells with hypodiploid DNA content after 16 hr are plotted in the middle panel. In the right panel, cells were lysed after 4 hr drug treatment for immunoblotting.

(B) MCF-7 cells that stably expressed the FLAG-Chk1-ER fusion protein were synchronized with a double-thymidine block. The cells were released into S phase for 8 hr, with or without 500 nM CPT, and in the absence or presence of 500 nM 4-OHT to induce the production of active FLAG-Chk1-ER. Samples were collected at the indicated times after release for cell cycle distribution analysis by flow cytometry. In the right panel, cells were harvested after 8 hr CPT treatment for immunoblot analysis. Numbers at the top of each sample lane represent the relative FLAG-Chk1-ER protein level, normalized to that obtained in non-CPT-treated samples.

(C) Asynchronous MCF-7 cells were cotransfected with empty vector, or Myc-Chk1 or HA-Chk2 expression plasmids, together with a membrane-anchored GFP expression vector. After 48 hr, the indicated samples were treated for 8 hr with 500 nM CPT. The cells were then cultured in fresh medium for an additional 16 hr. DNA contents in the GFP^+ and GFP^- cell populations were assayed by flow cytometry. The right panel shows expression levels of Chk1 and Chk2 at 48 hr posttransfection. Broad and narrow arrows represent ectopically expressed and endogenous proteins, respectively.

not in the untreated control cells. The latter results lend further support to the concept that the loss of Chk1 provoked by genotoxic agents is linked to Chk1 activation, and is not explained by the suppression of *CHK1* gene transcription.

The impact of FLAG-Chk1-ER activation on cellular recovery from CPT-induced S phase arrest was examined by treating the cells for 8 hr with CPT, in the absence or presence of 4-OHT. The cells were then transferred into culture medium without CPT, and cell cycle distribution was analyzed after 8 or 12 hr (Figure 2B, left panel). In the absence of 4-OHT, the FLAG-Chk1-ER-transfected cells remained stalled in S phase, consistent with the results obtained with the parental MCF-7 cell line (see Figure 2C and results not shown). In contrast, conditional induction of FLAG-Chk1-ER with 4-OHT allowed the cells to progress through S phase and into G_2-M phase. We also examined whether the cells recovered from CPT-induced stress when 4-OHT was added only *after* the CPT exposure period. In this case, only a minor subpopulation of cells progressed into G_2-M phase (results not shown), suggesting that delayed restoration of Chk1 activity cannot reverse established damage to DNA replication forks.

We next determined whether the rescue of S phase arrest was specific to Chk1 by cotransfecting MCF-7 cells with expression plasmids encoding either Chk1 or Chk2, together with membrane-anchored GFP as a marker for the transfected cell population. The cells were then treated for 8 hr with 500 nM CPT, and cell

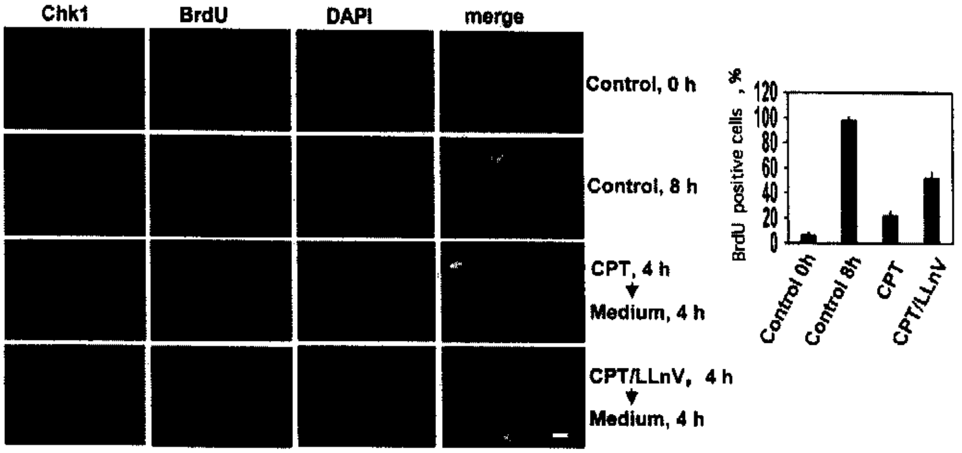

Figure 3. Role of Chk1 in Recovery from CPT-Induced Replication Stress

A549 cells were synchronized with a double-thymidine block, and released into S phase for 4 hr in the presence of 500 nM CPT, minus or plus LLnV. The cells were pulsed with BrdU during the final hour of this stage of culture. The drug-containing medium was then replaced with fresh medium. After 4 hr, cells were fixed and stained with α-Chk1 and α-BrdU antibodies. The scale bar represents 10 μm. In the right panel, a minimum of 200 cell nuclei from two independent experiments were examined to quantitate the percentage of BrdU-positive cells (mean ± standard deviation).

cycle profiles were examined after a 16 hr recovery period. As expected, cells transfected with the empty vector accumulated in S phase after CPT exposure, whereas the Chk1-transfected (GFP$^+$) cells recovered from the drug treatment and progressed into G_2-M phase (Figure 2C). In contrast, Chk2 overexpression failed to reverse the S phase arrest induced by CPT (Figure 2C), in spite of the fact that CPT triggered activation of endogenous Chk2 (see Figures 1A and 1B). Hence, resumption of DNA synthesis and S phase progression after CPT exposure is specifically dependent on the continued expression of the Chk1 kinase.

The correlation between loss of Chk1 and DNA replication arrest was examined at the single-cell level by immunofluorescence microscopy of BrdU-pulsed A549 cells. After a double-thymidine block, the cells were released into S phase for 4 hr in the presence of CPT, minus or plus LLnV, and were pulsed with BrdU during the last hour of drug treatment. The cells were then cultured for another 4 hr in drug-free medium and stained for Chk1- and BrdU-labeled DNA. As expected, virtually all nuclei stained positively for Chk1 and BrdU in the control sample (Figure 3). Treatment with CPT alone markedly reduced the numbers of Chk1-positive cell nuclei, and these nuclei also failed to incorporate BrdU into DNA. Interestingly, cotreatment with the proteasome inhibitor LLnV partially rescued both Chk1 immunoreactivity and BrdU incorporation in the drug-treated cells. Conversely, treatment with UCN-01 blocked BrdU incorporation, even in those cells that retained Chk1 after CPT treatment (results not shown). Collectively, these results demonstrate that loss of Chk1 protein and/or activity is causally related to the irreversible S phase arrest induced by CPT.

Genotoxic Stress-Induced Phosphorylation Targets Chk1 for Degradation

We noted that genotoxic stress-induced Chk1 downregulation was strongly correlated with the phosphorylation of this protein (Figure S4A). To further examine this relationship, we tested the effects of the PI 3-kinase-related kinase (PIKK) inhibitors, caffeine and wortmannin (Sarkaria et al., 1998, 1999), on CPT-induced Chk1 downregulation. Pretreatment with 10 μM wortmannin failed to antagonize the loss of Chk1 in the CPT-treated cells, whereas this response was effectively blocked by 10 mM caffeine (Figure 4A). Both drugs inhibit ATM activity under these conditions, whereas ATR activity is caffeine sensitive but wortmannin resistant (Sarkaria et al., 1998). Hence, these pharmacologic results implicated ATR in the CPT-induced destabilization of Chk1. To confirm this conclusion, we suppressed ATR and/or ATM expression with specific siRNAs. CPT-induced loss of Chk1 was clearly attenuated in the ATR-deficient cells, whereas ATM depletion had little effect on Chk1 downregulation in the drug-treated cells (Figure 4B). Collectively, these results suggest that phosphorylation of Chk1 by ATR not only activates Chk1, but also marks this protein for destruction by the proteasome.

ATR phosphorylates Chk1 at two Ser-Gln motifs (Ser-317 and Ser-345) in intact cells (Liu et al., 2000; Zhao and Piwnica-Worms, 2001). To examine the relative contributions of these phosphorylation sites to Chk1 degradation, we transfected HEK 293T cells with expression plasmids encoding wild-type or mutated Chk1 constructs containing Ala substitutions at either Ser-345 or Ser-317. Following treatment with CPT, expression levels of endogenous Chk1 were reduced to a similar extent in each of the transfected cell lines (Figure 4C; relevant bands indicated with the lower arrow). The levels of the Myc-tagged Chk1 proteins are most readily assessed in the top panel of Figure 4C (relevant bands are indicated with the broad arrow). CPT treatment caused an approximate 50% reduction in the levels of both the myc-Chk1 wild-type and S317A proteins, whereas the S345A mutant was considerably more resistant to CPT-induced downregulation. These results suggested that Ser-345 phosphorylation speci-

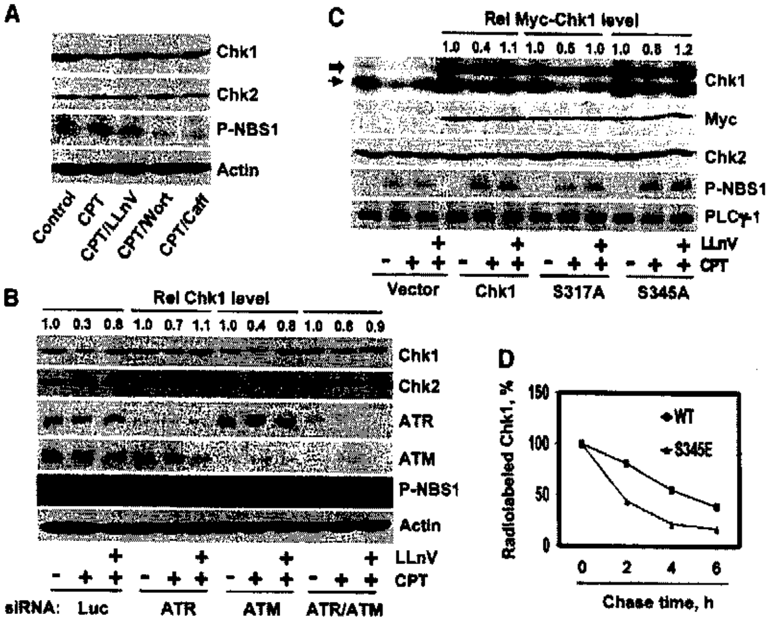

Figure 4. Role of ATR-Dependent Phosphorylation in Chk1 Degradation

(A) Asynchronous MCF-7 cells were pretreated for 20 min with 10 μM wortmannin (wort) or 10 mM caffeine (caff), and then were cultured for 8 hr in the presence of 500 nM CPT. Where indicated, LLnV was added during the final 4 hr of CPT treatment. Protein expression was analyzed by immunoblotting with the indicated antibodies.

(B) U2-OS cells were transfected with siRNAs targeted against luciferase (Luc), ATR, ATM, or ATR/ATM. After 48 hr, the cells were treated for 4 hr with CPT, followed by addition of LLnV to the indicated samples. After an additional 4 hr in culture, the cells were lysed for immunoblot analysis. Numbers at the top of each sample lane represent the Chk1 protein level, normalized to that obtained in the no-drug control for each cell population.

(C) HEK 293T cells were transfected with empty vector, or expression vectors encoding Myc-Chk1 wild-type, Myc-Chk1 S317A, or Myc-Chk1 S345A proteins. After 48 hr, the cells were treated for 8 hr with CPT and LLnV as described in (B). Protein expression was analyzed by immunoblotting. Thick and thin arrows represent ectopically expressed and endogenous Chk1 proteins, respectively. Numbers at the top of each sample lane represent the relative Myc-Chk1 protein level, normalized to that obtained in the no-drug control.

(D) HEK 293T cells were cotransfected with HA-tagged Chk1 wild-type or S345E expression plasmids. After 48 hr, the cells were metabolically labeled for 1 hr with ^{35}S-methionine/cysteine mixture and chased for the indicated time periods. Chk1 proteins were immunoprecipitated with α-HA antibodies, and separated by SDS-PAGE. ^{35}S-labeled Chk1 protein was quantitated by liquid scintillation counting, and radioactive counts were normalized to the 0 hr control.

fically promotes the destabilization of Chk1 during replication stress.

In subsequent studies, we compared the half-lives of wild-type Chk1 and a phospho-mimic (S345E) Chk1 mutant in pulse-chase experiments with radio-labeled cells. The HA-Chk1 wild-type protein declined with a half-life of approximately 3.8 hr, whereas the corresponding Chk1 S345E mutant displayed a half-life of only 1.6 hr (Figure 4D). Thus, the presence of a negatively charged residue at position 345 destabilizes the Chk1 polypeptide. We noted, however, that CPT exposure further enhanced the downregulation of the Chk1 S345E mutant (Figure S4B), suggesting that high-intensity replication stress triggers additional events that contribute to Chk1 destabilization.

Stress-Induced Chk1 Ubiquitination

We next determined whether Ser-345-phosphorylated Chk1 is modified by ubiquitination, a common prelude to proteasomal degradation. The abundance of ubiquitinated forms of Chk1 was strikingly increased in CPT-treated MCF-7 cells, particularly in the presence of the proteasome inhibitor LLnV (Figure 5A). The appearance of ubiquitinated Chk1 in cells treated with LLnV alone reinforces the idea that Chk1 is subject to continuous modification in cycling cells, likely due to activation of the replication checkpoint by intrinsic impediments to replication fork progression. In subsequent studies, we transfected MCF-7 cells with Myc-tagged Chk1 wild-type, Chk1 S317A, or Chk1 S345A constructs in order to determine whether either or both of the known ATR phosphorylation sites were required for Chk1 ubiquitination. Mutation of the Ser-345 but not the Ser-317 residue abolished the ubiquitination of ectopically expressed Chk1 in CPT-treated cells (Figure 5B).

To confirm that Chk1 is a direct target for ubiquitin ligases in mammalian cells, we performed in vitro ubiquitination reactions with ^{35}S-labeled Chk1 protein as the substrate. The wild-type and Chk1 S345E polypeptides underwent substantial ubiquitination in the presence of purified E1 and E2 proteins, together with A549 cell extract (Figure 5C and results not shown). In contrast, Chk1 (S345A) was a relatively poor substrate for ubiquitin ligases in these assays. To prove that Chk1 was directly ubiquitinated in intact cells, we transfected MCF-7 cells with an expression vector encoding polyhistidine ([His]$_6$)-tagged ubiquitin. The cells were exposed to CPT in the absence or presence of LLnV, and (His)$_6$-ubiquitin-bound proteins were captured under denaturing conditions with Ni^{2+}-agarose beads. Treatment of the cells with CPT or LLnV alone led to the appearance of modified forms of Chk1, as detected by immunoblotting with the α-Chk1 antibody, and combined treatment with both of these agents significantly increased the level of α-Chk1-reactive protein in these samples (Figure 5D). Collectively, these results demonstrate that Chk1 is inducibly and directly ubiquitinated in cells treated with CPT.

Cul1 and Cul4A Are Involved in Chk1 Degradation

Given the link between Chk1 phosphorylation and Chk1 ubiquitination, we considered one or more of the cullin-based E3 ligases as potential mediators of this response in human cells. Epitope-tagged versions of the six known human cullins (Kipreos et al., 1996) were transiently expressed in HEK 293T cells, which were

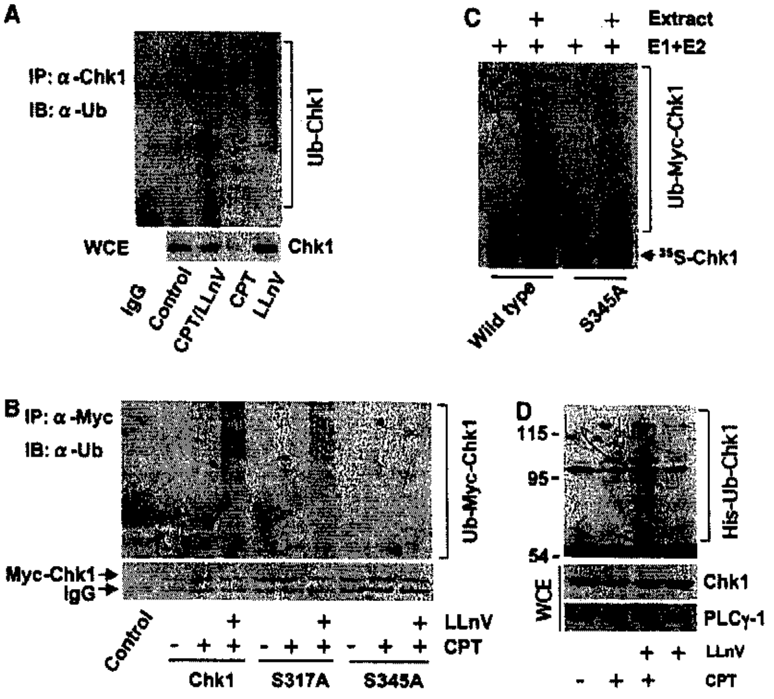

Figure 5. Genotoxic Stress-Induced Ubiquitination of Chk1

(A) Asynchronous MCF-7 cells were treated for 8 hr with 500 nM CPT, with LLnV added to the indicated samples during the final 4 hr in culture. Cell extracts were prepared and immunoprecipitated with α-Chk1 antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, and were immunoblotted with α-ubiquitin antibodies. Whole-cell extracts (WCE) were blotted with α-Chk1 antibodies.

(B) MCF-7 cells cotransfected with the indicated Chk1 expression vectors, together with a FLAG-tagged ubiquitin expression plasmid. After 48 hr, cells were treated with CPT and LLnV as described in (A). Cell extracts were immunoprecipitated with α-Myc antibodies. Protein blots were probed with α-ubiquitin antibodies, then stripped and reprobed with α-Myc antibodies.

(C) ^{35}S-labeled Chk1 proteins were subjected to in vitro ubiquitination reactions in the presence or absence of A549 cell extract as described in Experimental Procedures.

(D) MCF-7 cells were transfected with (His)$_6$-ubiquitin expression plasmid. After 48 hr, the cells were treated for 6 hr with 500 nM CPT and 4 μM LLnV. (His)$_6$-ubiquitin-bound proteins were captured with Ni^{2+}-agarose beads and analyzed by immunoblotting. Bottom panels: whole-cell extracts (WCE; 5% of total sample volume) were immunoblotted for total protein expression. The numbers on the left indicate molecular mass calibration standards (kD). Unmodified Chk1 comigrates with the 54 kDa calibration standard.

subsequently treated with CPT. Immunoblot analyses of α-Chk1 immunoprecipitates with tag-specific antibodies revealed that two cullin family members, Cul1 and Cul4A, were selectively coimmunoprecipitated with Chk1 under these conditions (Figure 6A). The association of both cullins with Chk1 increased in a time-dependent fashion during the first 4 hr of CPT treatment, after which time the amounts of Chk1 and coimmunoprecipitating Cul1 and Cul4A declined (Figure 6B). Cotransfection of the cells with Chk1 wild-type, S317A, or S345A, together with Cul1, revealed that the Ser-345 phosphorylation site was specifically required for association with Cul1 (Figure 6C). Due to technical limitations, we were not able to examine the interaction between the ectopically expressed Cul4A and Chk1 proteins. Experiments with nontransfected cells confirmed that CPT exposure induced the association of endogenous Chk1 with Cul1 and Cul4A, but not with Cul3, in the presence of genotoxic stress (Figure 6D).

We next examined the effect of Cul1 or Cul4A depletion on replication stress-induced Chk1 degradation in siRNA-transfected U2-OS cells. A 60%–80% reduction in Cul1 or Cul4A expression with siRNA partially reversed the decrease in Chk1 protein triggered by CPT exposure, and simultaneous knockdown of both cullins had roughly additive effects on CPT-induced Chk1 degradation (Figure 6E). The cullin-depleted cell populations exhibited normal cell cycle distributions, indicating that the altered responses to CPT were not explained by nonspecific effects of Cul1 or Cul4A deficiency on S phase entry or progression (data not shown). Interestingly, we routinely observed that simultaneous depletion of Cul1 and Cul4A caused an increase in basal Chk1 expression in these cells, suggesting that these cullins also participate in Chk1 turnover during an otherwise unperturbed cell cycle (Figure S5).

Efforts to reconstitute Cul1/Cul4A-dependent ubiquitination of Chk1 in a cell-free system have thus far proven unsuccessful (our unpublished results). Consequently, we adopted a more indirect approach involving overexpression of Cul1 and/or Cul4A subunits in HEK 239T cells. The transfected cells were treated with CPT and LLnV to promote the accumulation of ubiquitinated forms of Chk1. Overexpression of either Cul1 or Cul4A clearly increased the level of ubiquitinated Chk1 in these cells, and coexpression of both cullins caused an additive increase in Chk1 ubiquitination (Figure 6F). The responses to Cul1 and Cul4A overexpression were specific, in that overexpression of Cul4B failed to enhance ubiquitination of the Chk1 protein. These results suggest that the ubiquitination of Chk1 is carried out by two SCF (Skp-Cullin-*F* box protein)-type E3 ligases containing either Cul1 or Cul4A.

Subcellular Localization of Chk1 during Replication Stress

To further probe the mechanism of replication stress-induced Chk1 degradation, we subjected A549 cells to an in situ fractionation protocol at various times after CPT exposure (Figure 7A). In untreated cells, substantial amounts of Chk1, as well as ATR, were present in the chromatin-enriched (CE) fraction. Treatment of the cells with CPT caused a rapid increase in Chk1 (Ser-345) phosphorylation that preceded the onset of Chk1 degradation (compare Figure 7A and Figure S1B). The increase in Chk1 phosphorylation coincided with the release of Chk1 from the chromatin-bound fraction, and

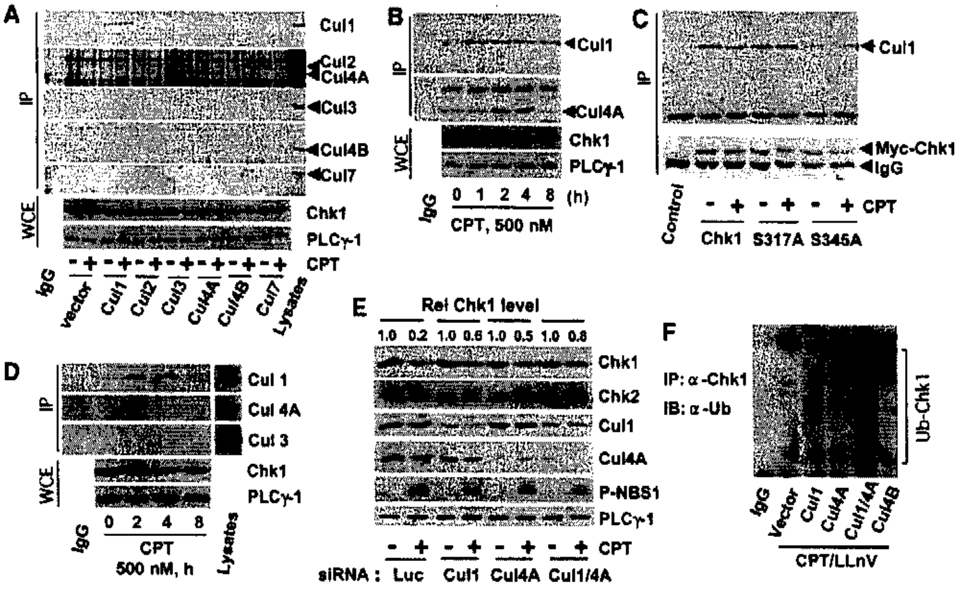

Figure 6. Cullin-Containing E3 Ligases Mediate Chk1 Degradation

(A) HEK 293T cells were transfected with HA-tagged Cul1 and Cul7, T7-tagged Cul2 and Cul4A, Cul3, or V5-tagged Cul4B expression plasmids. After 48 hr, the cells were treated for 8 hr with 500 nM CPT. Cell extracts were immunoprecipitated (IP) with α-Chk1 antibodies, and proteins were immunoblotted with the appropriate α-tag or α-Cul3 antibodies. The two lower panels show immunoblots from whole-cell extracts (WCE) as a measure of total protein expression.

(B) HEK 293T cells were transfected with HA-Cul1 or T7-Cul4A. After 48 hr, the cells were treated with 500 nM CPT for the indicated times. Cell extracts were immunoprecipitated with α-Chk1 antibodies, and the resolved proteins were immunoblotted with α-HA or α-T7 antibodies. Total protein expression in WCE is shown in the two lower panels.

(C) HEK 293T cells were cotransfected with HA-Cul1 and Myc-Chk1 (wild-type, S317A, or S345A) expression plasmids. After 48 hr, the cells were treated for 8 hr with 500 nM CPT. Cell extracts were immunoprecipitated with α-Myc and immunoblotted with α-HA antibodies. The membrane was stripped and reblotted with anti-Myc antibodies.

(D) Asynchronous MCF-7 cells were treated with 500 nM CPT for the indicated times, and cell extracts were immunoprecipitated with mouse IgG (negative control) or α-Chk1 antibodies. Proteins were immunoblotted with α-Cul4A antibodies, and the membrane was stripped and reprobed with the α-Cul1 or α-Cul3 antibodies. WCE were immunoblotted to indicate total protein expression.

(E) U2-OS cells were transfected with the indicated siRNAs. After 48 hr, the cells were treated for 8 hr with 500 nM CPT, and cellular proteins were immunoblotted with the indicated antibodies. Numbers at the top of each sample lane represent the relative Chk1 protein level, normalized to that obtained in the no-drug control.

(F) HEK 293T cells were transfected with expression plasmids encoding HA-Cul1, T7-Cul4A, or V5-Cul4B, together with a FLAG-tagged ubiquitin expression plasmid. After 48 hr, the cells were treated for 4 hr with 500 nM CPT, followed by addition of 2 μM LLnV. After an additional 4 hr in culture, cell extracts were prepared and immunoprecipitated with α-Chk1 antibodies. Immunoprecipitated proteins were immunoblotted with α-ubiquitin antibodies.

the appearance of the phosphorylated protein in fractions enriched for soluble nuclear (Nu-S) and cytoplasmic (Cyto) proteins. In contrast, ATR remained in the CE fraction throughout the time course of CPT exposure. The behavior of the Chk2 protein differed markedly from that of Chk1. Chk2 was present mainly in the Nu-S fraction in untreated cells, and gradually redistributed into the cytoplasmic compartment after 2 hr of CPT treatment.

To confirm these results, we examined the effects of CPT on Chk1 subcellular localization, in the presence of LLnV to suppress Chk1 degradation. In untreated A549 cells, Chk1 was localized predominantly in the nucleus (Figure 7B). Treatment with LLnV for 1–2 hr resulted in the appearance of scattered cells that displayed prominent cytoplasmic staining for Chk1, consistent with previous evidence that Chk1 is activated and degraded at low levels during the normal cell cycle. Treatment of the cells with LLnV plus CPT led to the accumulation of cytoplasmic Chk1 in many cells, indicating that proteasomal degradation normally suppresses this event in cells exposed to high-intensity replication stress.

We determined whether ubiquitinated forms of Chk1 accumulated in the nucleus and/or cytoplasmic fractions of CPT-treated cells. After CPT exposure, ubiquitinated Chk1 was found in the Nu-S fraction as well as the Cyto fraction of CPT-treated cells (Figure 7C). Furthermore, extracts from both the Nu-S and Cyto compartments supported in vitro ubiquitination of Chk1 (data not shown). Interestingly, Chk1-associated Cul1 was found predominantly in the Nu-S fraction, and multiple Cul1-immunoreactive bands were coimmunoprecipitated with Chk1 from this fraction (Figure 7D, top panel). These immunoreactive bands apparently represent modified forms of Cul1, because all three bands were eliminated in Cul1 siRNA-transfected cells (our unpublished results). Unlike Cul1, Cul4A was expressed at comparable levels in the Cyto and Nu-S fractions, and Chk1-associated Cul4A was found mainly in the cytoplasmic fraction (Figure 7D). These results indicate

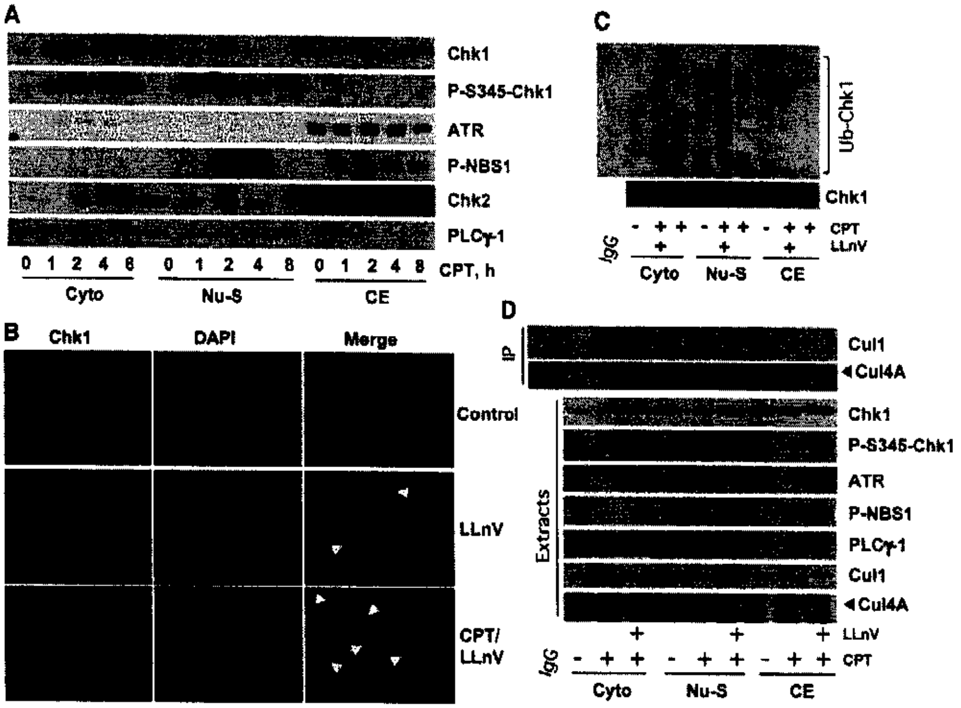

Figure 7. Replication Stress-Induced Chk1 Subcellular Redistribution

(A) Asynchronous A549 cells were treated with 500 nM CPT for the indicated times, and the cells were sequentially extracted to yield cytoplasmic (Cyto), soluble nuclear (Nu-S), and chromatin-enriched (CE) fractions.

(B) Asynchronous A549 cells were treated for 1.5 hr with 4 μM LLnV, or with LLnV plus 500 nM CPT. The cells were then fixed and stained with anti-Chk1 antibodies. The scale bar represents 10 μm. Arrows denote cells staining positively for cytoplasmic Chk1.

(C) Asynchronous A549 cells were treated for 4 hr with 500 nM CPT, and 4 μM LLnV was added to the indicated samples. After 4 hr, the cells were fractionated as described in (A), and fractions were immunoprecipitated with α-Chk1 antibodies, followed by immunoblotting with α-ubiquitin antibodies. In the lower panel, whole-cell extracts were immunoblotted directly with α-Chk1 antibodies.

(D) Asynchronous A549 cells were treated for 4 hr with 500 nM CPT minus or plus 4 μM LLnV. The cells were then sequentially extracted, and the subcellular fractions were immunoprecipitated with α-Chk1 antibodies, followed by immunoblotting with α-Cul4A and α-Cul1 antibodies. Parallel cell extracts were also immunoblotted with the indicated antibodies.

that replication stress triggers Chk1 phosphorylation at Ser-345, leading to dynamic changes in Chk1 subcellular localization, and, in turn, to the ubiquitination of the phosphorylated protein by at least two cullin-containing E3 ligases.

Discussion

The present findings have significant implications for replication checkpoint control, and for the mechanisms of action of some clinically important anticancer drugs. ATR is well established as the upstream kinase that phosphorylates and activates Chk1 in response to replicative stress (Bartek et al., 2004). The present results indicate that ATR-mediated phosphorylation of Chk1 at Ser-345 not only leads to Chk1 activation, but also marks this protein for eventual destruction by the ubiquitin-proteasome pathway. This study follows on the heels of a report identifying the oncogenic phosphatase WIP1/PPMID as a Chk1-regulatory enzyme that specifically dephosphorylates the Ser-345 residue (Lu et al., 2005). Collectively, these studies implicate Ser-345 as a nodal site for the regulation of Chk1 activation and inactivation in human cells.

During a normal S phase, the ATR-Chk1 pathway continuously monitors the progress of DNA replication forks, and suppresses the firing of later replication origins when fork progression is impaired (e.g., during replication through fragile sites) (Feijoo et al., 2001; Shechter et al., 2004). We speculate that coupling the activation of Chk1 to the subsequent destabilization of this protein prevents the activated kinase from accumulating and inappropriately delaying DNA replication. In the setting of prolonged and/or high-intensity replicative stress, however, Chk1 degradation may be so extensive that stalled replication forks are irreversibly damaged, resulting in permanent S phase arrest and ultimately cell death. A recent study demonstrates that a partial reduction in Chk1 expression (in this case due to reduced gene dosage) is sufficient to impair both S phase progression and cell survival during normal tissue development in vivo (Lam et al., 2004).

Previous reports indicate that *CHK1* gene transcription varies during the normal cell cycle (Kaneko et al., 1999), and in cells exposed to genotoxic stress (Gottifredi et al., 2001). Our studies uncovered a distinct posttranscriptional pathway of Chk1 regulation in which replication stress itself induces the proteolysis of Chk1. As stated above, the targeted destruction of activated Chk1 may self-limit the duration of ATR-dependent S phase checkpoint signaling during the normal cell cycle. Along these lines, we noted that the degradation of Chk1 is temporally delayed relative to the phosphorylation of this protein by ATR. This delay presumably allows activated Chk1 to carry out its checkpoint signaling functions before its removal by ubiquitination and proteolysis. Our cell fractionation experiments suggest that the temporal delay between Chk1 activation and the onset of Chk1 ubiquitination is determined in part by the time required for the mobilization of activated Chk1 from chromatin to the soluble nuclear compartment, where the protein kinase first encounters Cul1/Cul4A-containing E3 ligases.

Earlier studies identified an alternative pathway that limits the duration of ATR-Chk1 signaling and promotes cellular recovery from DNA damage incurred prior to M phase. The adaptor protein, claspin, is involved in the coupling of ATR activation to the phosphorylation of Chk1 in cells exposed to replication stress (Sorensen et al., 2004). Studies in *Xenopus* indicate that phosphorylation of claspin by the polo-like kinase Plx1 disrupts communication between ATR and Chk1, resulting in checkpoint adaptation and cell cycle progression into mitosis (Yoo et al., 2004). If this mechanism of Chk1 inactivation exists in mammalian cells, its primary function may be to promote G_2 to M phase progression after the complete resolution of DNA damage.

The mechanisms through which Ser-345 phosphorylation regulates Chk1 function and stability are only partially understood. Phosphorylation of this residue may relieve an autoinhibitory influence on the catalytic domain, thereby increasing Chk1 kinase activity (Katsuragi and Sagata, 2004). Our findings suggest that Ser-345 phosphorylation unmasks a cryptic degron motif that sets in motion the events leading to Chk1 ubiquitination. Identification of the F box proteins that mediate binding of phosphorylated Chk1 to Cul1- and Cul4A-containing E3 ligases will fill a major conceptual gap in our understanding of Chk1 regulation. The mammalian genome encodes more than 60 F box proteins (Jin et al., 2004), and genetic screens aimed toward the delineation of the specific family members involved in replication stress-induced Chk1 downregulation are now underway.

Although the mechanisms of action of CPT and other Top1 poisons have been studied extensively, the determinants of cancer cell sensitivity to these drugs are poorly understood. Collisions between active replication forks and DNA-bound CPT-Top1 complexes interrupt fork progression and induce DNA double-strand breaks (Hsiang et al., 1989). Predictably, one route to CPT resistance in human tumors involves a reduction in Top1 activity, through either mutation or reduced expression of Top1 (Rasheed and Rubin, 2003). However, accumulating evidence suggests that the ATR-Chk1 pathway is centrally involved in recovery from Top1 poison-induced replicative stress (Cliby et al., 2002; Pommier et al., 2003). Cancer cells, particularly those residing in hypoxic regions of solid tumors, may rely heavily on this checkpoint pathway for genome surveillance and repair during S phase (Hammond and Giaccia, 2004). We propose that the favorable therapeutic index of CPT and related agents in human cancer patients derives, in part, from their ability to trigger the degradation of the cytoprotective Chk1 protein in tumor cells that are coping with chronic replication stress imposed by the tumor microenvironment.

Experimental Procedures

Cell Culture and Antibodies

A549 cells were cultured in DMEM/F12 with 10% FBS. MDA-MB-231 cells were cultured in α-MEM with 10% FBS, 1 mM sodium pyruvate, MEM vitamin solution, and 10 mM glutamine. MCF-7 and 293T cells were grown in DMEM (high-glucose) with 10% FBS, and U2-OS cells were cultured in DMEM (low-glucose) supplemented with 10% FBS. Human diploid BJ fibroblasts were cultured in DMEM (high-glucose) with 20% FBS.

Antibodies against Chk1 (G4), Rad17, and Cul4 (H-66, C-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ATM (Ab-3) and α-ATR (Ab-1) were obtained from Oncogene Science Research Products (Cambridge, MA). Anti-Chk2 antibody was obtained from Upstate Biotechnology (Waltham, MA). Anti-Cul1 and α-Cul3 antibodies were from Invitrogen (Carlsbad, CA). Rat α-HA (3F10) polyclonal antibody was from Roche Molecular Biochemicals (Indianapolis, IN) and α-phospho-histone H3 was from Cell Signaling Technology (Beverly, MA).

Phosphatase Treatment

CPT- or MMS-treated cells were lysed in 25 mM HEPES, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors. The cleared extracts were treated with 600 units of λ protein phosphatase (New England BioLabs, Ipswich, MA) in the presence of Mn^{2+} for 1 hr at 37°C. The reaction was stopped with 2× SDS-PAGE sample buffer and boiled for 5 min. Extracts were run on SDS-PAGE gels for immunoblotting.

Transfection of siRNA

Synthetic siRNAs were purchased from Dharmacon (Lafayette, CO). The Chk1 siRNA sequence was reported previously (Zhao et al., 2002). The siRNA sequences used for each target protein were: ATM, 5′-AAGCACCAGUCCAGUAUUGGC-3′; ATR, 5′-AACGAGACUUCUGCGGAUUGC-3′; Cul1, 5′-AACGAAGAGUUCAGGUUUACC-3′. The Dharmacon siGENOME SMART pool Upgrade was used for Cul4A gene silencing. Transfection of siRNAs was performed as previously described (Brumbaugh et al., 2004).

Chk1 Constructs

The Chk1 mutants, S317A and S345A, were described previously (Zhao and Piwnica-Worms, 2001). The S345E mutation of Chk1 was performed with the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the HA-tagged Chk1 wild-type plasmid as the template.

To generate the 4-OHT-inducible FLAG-Chk1-ER fusion protein, the human Chk1 cDNA was amplified by PCR and simultaneously appended with 5′ nucleotides encoding the FLAG epitope. The PCR product was cloned into a pBluescript plasmid containing the mutated ER (G525R) cDNA (Littlewood et al., 1995). The resulting FLAG-Chk1-ER insert was excised with EcoR1, and the insert was cloned into the pcDNA 3.1(+) expression plasmid. The fidelity of the PCR and cloning procedures was verified by nucleotide sequencing.

Ubiquitination Assays

For in vitro ubiquitination reactions, ^{35}S-labeled Chk1 protein was produced by in vitro transcription-translation, and ubiquitination reactions were performed in the presence of A549 cell extracts (Carrano et al., 1999). Briefly, A549 cells were lysed by sonication in 20 mM Tris-HCl (pH 7.2), 2 mM DTT, 0.25 mM EDTA, and protease inhibitors. The lysates were cleared by centrifugation, the protein content was assayed, and the supernatants were aliquoted and stored at −80°C. Ubiquitination reactions were performed in 10–30 μl samples containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 5 μM MG-132, 50 ng E1, 200 ng E2, 5 μM ubiquitin, 10 mM phosphocreatine, 0.1 μg/μl creatine phosphokinase, 1 mM ATP, 30 μg cell extract, and 1 μl of unpurified, ^{35}S-labeled

Myc-Chk1 or the same protein purified by α-Myc immunoprecipitation. Reactions were performed for 1 hr at 30°C, terminated with 2× sample buffer, and denatured proteins were separated by SDS-PAGE. Ubiquitinated Chk1 proteins were identified by autoradiography.

For in vivo ubiquitination studies, MCF-7 cells were transfected with $(His)_6$-ubiquitin plasmid, and, after 24 hr, the cells were treated for 4–6 hr with 500 nM CPT in the presence or absence of 4 μM LLnV. The cells were lysed in 6 M guanidinium hydrochloride, and soluble proteins were precipitated with Ni^{2+}-agarose beads. The bound proteins were eluted from Bio-Rad Poly-Prep chromatography columns (Hercules, CA), run on SDS-PAGE gels, and immunoblotted with α-Chk1 antibodies.

Biochemical Fractionation, Immunoblotting, and Immunoprecipitation

Subcellular fractions were prepared as previously described (Mendez and Stillman, 2000). Cell extraction, immunoblotting, and immunoprecipitation were performed as described in Brumbaugh et al. (2004).

Supplemental Data

Supplemental figures can be found with this article online at http://www.molecule.org/cgi/content/full/19/5/607/DC1/.

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References

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. *15*, 2177–2196.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat. Rev. Mol. Cell Biol. *5*, 792–804.

Brumbaugh, K.M., Otterness, D.M., Geisen, C., Oliveira, V., Brognard, J., Li, X., Lejeune, F., Tibbetts, R.S., Maquat, L.E., and Abraham, R.T. (2004). The mRNA surveillance protein hSMG-1 functions in genotoxic stress response pathways in mammalian cells. Mol. Cell *14*, 585–598.

Busby, E.C., Leistritz, D.F., Abraham, R.T., Karnitz, L.M., and Sarkaria, J.N. (2000). The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. Cancer Res. *60*, 2108–2112.

Carrano, A.C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. *1*, 193–199.

Casper, A.M., Nghiem, P., Arlt, M.F., and Glover, T.W. (2002). ATR regulates fragile site stability. Cell *111*, 779–789.

Cha, R.S., and Kleckner, N. (2002). ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science *297*, 602–606.

Cliby, W.A., Lewis, K.A., Lilly, K.K., and Kaufmann, S.H. (2002). S phase and G(2) arrests induced by topoisomerase I poisons are dependent on ATR kinase function. J. Biol. Chem. *277*, 1599–1606.

Feijoo, C., Hall-Jackson, C., Wu, R., Jenkins, D., Leitch, J., Gilbert, D.M., and Smythe, C. (2001). Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. J. Cell Biol. *154*, 913–923.

Gottifredi, V., Karni-Schmidt, O., Shieh, S.S., and Prives, C. (2001). p53 down-regulates CHK1 through p21 and the retinoblastoma protein. Mol. Cell. Biol. *21*, 1066–1076.

Hammond, E.M., and Giaccia, A.J. (2004). The role of ATM and ATR in the cellular response to hypoxia and re-oxygenation. DNA Repair (Amst.) *3*, 1117–1122.

Hsiang, Y.H., Lihou, M.G., and Liu, L.F. (1989). Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. Cancer Res. *49*, 5077–5082.

Jin, J., Cardozo, T., Lovering, R.C., Elledge, S.J., Pagano, M., and Harper, J.W. (2004). Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev. *18*, 2573–2580.

Kaneko, Y.S., Watanabe, N., Morisaki, H., Akita, H., Fujimoto, A., Tominaga, K., Terasawa, M., Tachibana, A., Ikeda, K., Nakanishi, M., and Kaneko, Y. (1999). Cell-cycle-dependent and ATM-independent expression of human Chk1 kinase. Oncogene *18*, 3673–3681.

Katsuragi, Y., and Sagata, N. (2004). Regulation of Chk1 kinase by autoinhibition and ATR-mediated phosphorylation. Mol. Biol. Cell *15*, 1680–1689.

Kipreos, E.T., Lander, L.E., Wing, J.P., He, W.W., and Hedgecock, E.M. (1996). cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family. Cell *85*, 829–839.

Lam, M.H., Liu, Q., Elledge, S.J., and Rosen, J.M. (2004). Chk1 is haploinsufficient for multiple functions critical to tumor suppression. Cancer Cell *6*, 45–59.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *23*, 1686–1690.

Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., et al. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. *14*, 1448–1459.

Lu, X., Nannenga, B., and Donehower, L.A. (2005). PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. Genes Dev. *19*, 1162–1174.

Mendez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol. Cell. Biol. *20*, 8602–8612.

Pommier, Y., Redon, C., Rao, V.A., Seiler, J.A., Sordet, O., Takemura, H., Antony, S., Meng, L., Liao, Z., Kohlhagen, G., et al. (2003). Repair of and checkpoint response to topoisomerase I-mediated DNA damage. Mutat. Res. *532*, 173–203.

Rasheed, Z.A., and Rubin, E.H. (2003). Mechanisms of resistance to topoisomerase I-targeting drugs. Oncogene *22*, 7296–7304.

Rivory, L.P., Haaz, M.C., Canal, P., Lokiec, F., Armand, J.P., and Robert, J. (1997). Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. Clin. Cancer Res. *3*, 1261–1266.

Sarkaria, J.N., Tibbetts, R.S., Busby, E.C., Kennedy, A.P., Hill, D.E., and Abraham, R.T. (1998). Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res. *58*, 4375–4382.

Sarkaria, J.N., Busby, E.C., Tibbetts, R.S., Roos, P., Taya, Y., Karnitz, L.M., and Abraham, R.T. (1999). Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res. *59*, 4375–4382.

Shechter, D., Costanzo, V., and Gautier, J. (2004). ATR and ATM regulate the timing of DNA replication origin firing. Nat. Cell Biol. *6*, 648–655.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer *3*, 155–168.

Siu, W.Y., Lau, A., Arooz, T., Chow, J.P., Ho, H.T., and Poon, R.Y. (2004). Topoisomerase poisons differentially activate DNA damage checkpoints through ataxia-telangiectasia mutated-dependent and -independent mechanisms. Mol. Cancer Ther. *3*, 621–632.

Sorensen, C.S., Syljuasen, R.G., Lukas, J., and Bartek, J. (2004). ATR, claspin and the Rad9-Rad1-Hus1 complex regulate Chk1 and Cdc25A in the absence of DNA damage. Cell Cycle *3*, 941–945.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y.A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakanishi, M., and Nakayama, K. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(–/–) mice. Genes Dev. *14*, 1439–1447.

Yoo, H.Y., Kumagai, A., Shevchenko, A., and Dunphy, W.G. (2004). Adaptation of a DNA replication checkpoint response depends upon inactivation of claspin by the polo-like kinase. Cell *117*, 575–588.

Zachos, G., Rainey, M.D., and Gillespie, D.A. (2003). Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. EMBO J. *22*, 713–723.

Zhao, H., and Piwnica-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol. Cell. Biol. *21*, 4129–4139.

Zhao, H., Watkins, J.L., and Piwnica-Worms, H. (2002). Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc. Natl. Acad. Sci. USA *99*, 14795–14800.

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Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling

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Human Rad17 (hRad17) is centrally involved in the activation of cell-cycle checkpoints by genotoxic agents or replication stress. Here we identify hMCM7, a core component of the DNA replication apparatus, as a novel hRad17-interacting protein. In HeLa cells, depletion of either hRad17 or hMCM7 with small-interfering RNA suppressed ultraviolet (UV) light- or aphidicolin-induced hChk1 phosphorylation, and abolished UV-induced S-phase checkpoint activation. Similar results were obtained after transfection of these cells with a fusion protein containing the hMCM7-binding region of hRad17. The hMCM7-depleted cells were also defective for the formation of ATR-containing nuclear foci after UV irradiation, suggesting that hMCM7 is required for stable recruitment of ATR to damaged DNA. These results demonstrate that hMCM7 plays a direct role in the transmission of DNA damage signals from active replication forks to the S-phase checkpoint machinery in human cells.

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Keywords: ATR; DNA replication; hChk1; MCM proteins

Introduction

Genomic integrity in eukaryotic cells is continuously challenged by DNA-damaging agents generated as by-products of normal cellular metabolism or derived from environmental sources. To maintain the stability of their genomes, cells have evolved a battery of DNA damage response pathways, termed cell-cycle checkpoints, which are differentially activated in response to distinct types of genotoxic stress (e.g., DNA double-strand breaks (DSBs), ultraviolet (UV)-induced photoproducts, or abnormal replication forks). Defects in checkpoint signaling lead to genetic instability, heightened sensitivity to DNA-damaging agents, and increased susceptibility to cancer development in mammals (Elledge, 1996; Storchova and Pellman, 2004).

The process of DNA replication is particularly sensitive to genotoxic stress, and requires continuous surveillance in order to ensure that duplication of the genome is executed with high fidelity during S phase. Loss or malfunction of proteins that monitor DNA replication fidelity, including ATR, hRad17, and hChk1, leads to genomic instability and cell death, even in the absence of extrinsic genotoxic agents (Brown and Baltimore, 2000; Wang *et al*, 2003). Recently, the mechanisms whereby S-phase cells detect and respond to damaged or aberrantly structured DNA have come under intensive scrutiny. Studies in yeast and *Xenopus* demonstrated that interfering with replication fork progression activates the S-phase checkpoint, and that mutations in the replication machinery led to defective S-phase checkpoint signaling (Lupardus *et al*, 2002; Stokes *et al*, 2002; Osborn and Elledge, 2003; Tercero *et al*, 2003). Although the underlying mechanisms are not well defined, mammalian cells also mount strong checkpoint responses to lesions that impede replication fork progression, such as UV-induced cyclobutane–pyrimidine dimers (Heffernan *et al*, 2002). Compelling evidence implicates two phosphatidylinositol 3-kinase-related kinases, ATR and ATM, as proximal transducers in S-phase checkpoint signaling pathways in mammalian cells (Abraham, 2001; Shiloh, 2003; Shechter *et al*, 2004). ATM is activated in response to DNA DSBs, which trigger specific alterations in histone structure (Bakkenist and Kastan, 2003), or the loading of the MRE11–Rad50–NBS1 complex at the site of strand breakage (Carson *et al*, 2003; Uziel *et al*, 2003; Lee and Paull, 2004). In contrast, ATR, together with its putative regulatory subunit, ATRIP (Cortez *et al*, 2002), is attracted to single-stranded DNA (ssDNA) coated with replication protein A (RPA) (Zou and Elledge, 2003), which is a common intermediate produced during the processing of damaged DNA. Accumulating evidence suggests that checkpoint signaling through ATR is intimately linked to the process of DNA replication (Hekmat-Nejad *et al*, 2000; Michael *et al*, 2000; Casper *et al*, 2002; Lupardus *et al*, 2002; Tercero *et al*, 2003; Marheineke and Hyrien, 2004; Shechter *et al*, 2004; Ward *et al*, 2004a, b).

During G_1 phase of the cell cycle, replication origins in DNA are licensed by the assembly of a pre-replication complex (pre-RC) comprising the origin recognition complex (ORC), Cdc6, and MCM (*m*ini*c*hromosome *m*aintenance) complex (Waga and Stillman, 1998; Tye, 1999). Upon entry of the cell into S phase, pre-RCs are converted into active replication forks by phosphorylation events carried out by the cyclin E–cdk2 and Dbf4–Cdc7 kinases. During fork progression through duplex DNA, unwinding and denaturation of the DNA is required to create a template for the replicative polymerases. The hexameric MCM complex, consisting of the hMCM2–7 proteins, is believed to function as a replicative helicase that mediates unwinding of DNA at origins of replication, as well as DNA in the path of active replication forks (You *et al*, 1999, 2002; Labib *et al*, 2000; Lei and Tye, 2001).

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Previous studies demonstrated physical and functional interactions between ATR and the key checkpoint protein hRad17 during the cellular response to damaged DNA or replication stress (Bao *et al*, 2001; Stokes *et al*, 2002; Zou *et al*, 2002; Wang *et al*, 2003). DNA damage incurred during G_1, S, or G_2 phase of the cell cycle is sensed by hRad17, which, acting in a complex with the replication factor C (RFC) 2–5 subunits, loads three PCNA-related proteins, hRad9, hHus1, and hRad1 (termed the '9-1-1 complex'), near the damaged site (Burtelow *et al*, 2000; Bermudez *et al*, 2003). Although the ATRIP-ATR and 9-1-1 complexes are recruited independently to DNA damage sites (Kondo *et al*, 2001; Melo *et al*, 2001; Zou *et al*, 2002), the phosphorylation of many ATR substrates, including hChk1 and hRad17 itself, is highly dependent on the 9-1-1 complex (Zou *et al*, 2002; Jiang *et al*, 2003).

To further understand the checkpoint signaling functions of hRad17, we performed a yeast two-hybrid screen aimed toward the identification of novel hRad17-interacting proteins. In this study, we show that the MCM protein hMCM7 is a functionally important binding partner for hRad17 in human cells. The present findings indicate that, in addition to its contributions to normal DNA replication, hMCM7 is centrally involved in the activation of the ATR-dependent S-phase checkpoint by agents that induce DNA replication stress.

Results

Using full-length hRad17 as the bait, we performed a yeast two-hybrid screen with a human fetal brain cDNA library as the prey. Of the 33 positive clones that emerged from this screen, one encoded the carboxyl-terminal region of hMCM7, a component of the preinitiation complex assembled at origins of DNA replication (Waga and Stillman, 1998). To verify the yeast two-hybrid results, we cotransfected HeLa cells with HA-tagged hMCM7 and FLAG-tagged hRad17, and observed a significant amount of FLAG-hRad17 in the α-HA immunoprecipitates from these cells (Figure 1A, upper panel). The converse co-immunoprecipitation experiment revealed a modest but readily detectable amount of α-HA immunoreactivity in FLAG-Rad17 immunoprecipitates from the doubly transfected cells.

In subsequent experiments, we demonstrated that α-hRad17 antibodies co-precipitated endogenous hMCM7 from nontransfected HeLa cells (Figure 1B, left panel). Interestingly, exposure of cells to aphidicolin (Aph) or UV light increased the amount of hMCM-7 that co-immunoprecipitated with the endogenous hRad17. This experiment was repeated with three additional human cell lines (U2OS, A549, and human embryonic kidney 293T), and yielded similar results (not shown). Based on the prediction that ATR and its partner protein, ATRIP, reside in close proximity to the replication fork, we tested whether α-ATRIP immunoprecipitates also contained hMCM7 and/or hRad17. Both proteins were indeed present in the α-ATRIP immunoprecipitates, and, once again, the associations were increased by Aph or UV light exposure (Figure 1B, lower panel). Collectively, these results suggest that hMCM7 is closely associated with both ATRIP-ATR and hRad17 in both the absence and presence of agents that induce replication stress.

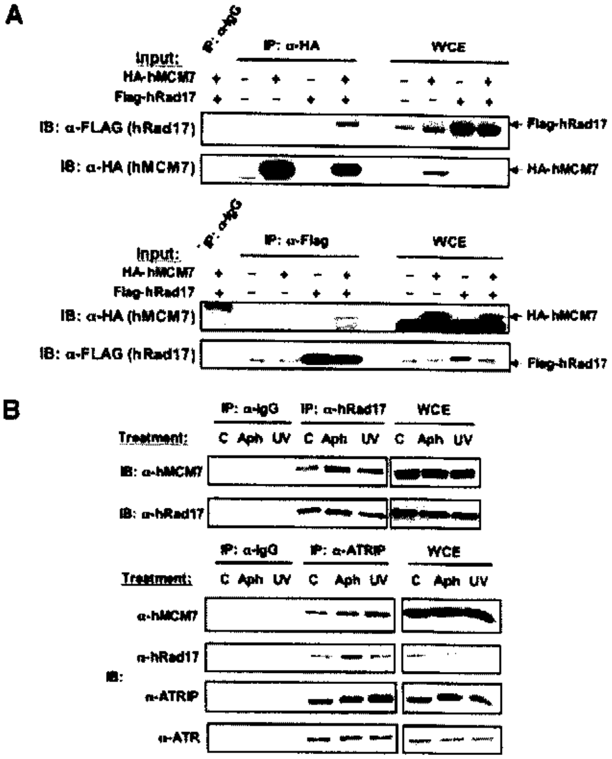

Figure 1 Co-immunoprecipitation of hRad17 with hMCM7. (A) Co-immunoprecipitation of FLAG-tagged hRad17 and HA-tagged hMCM7. HeLa cells were cotransfected with the indicated plasmids, and cellular extracts (0.5 mg protein) were immunoprecipitated (IP) with α-FLAG or α-HA antibodies. (B) Genotoxic stress-induced co-immunoprecipitation of hRad17, hMCM7, and ATRIP. HeLa cells were exposed for 4 h to 200 J/m^2 UV-B light or 1 μM Aph. Cellular extracts (1 mg protein) were immunoprecipitated and immunoblotted with the indicated antibodies.

Treatment of cells with inhibitors of DNA replication or UV light activates the ATR-dependent S-phase checkpoint (Abraham, 2001; Shiloh, 2003). To determine whether the interaction between hRad17 and hMCM7 was involved in genotoxic stress-induced ATR activation, we silenced hRad17 or hMCM7 gene expression in HeLa cells by transfection with specific siRNAs. These reagents reduced the expression of their respective target proteins by at least 80%, relative to the control cells that received luciferase (Luc)-specific siRNA. Both hRad17- and hMCM7-depleted cells displayed significant defects in hChk1 phosphorylation at Ser-345 after UV light exposure (Figure 2A). Furthermore, reduced expression of hMCM7 impaired UV-induced phosphorylation of the Ser-635 site in hRad17. These modifications of hChk1 and hRad17 are mediated by ATR in UV-damaged cells (Liu *et al*, 2000; Bao *et al*, 2001; Bartek and Lukas, 2003). Loss of hMCM7 did not lead to global defects in checkpoint signaling, as the phosphorylation of hChk2 provoked by ionizing radiation (IR) was not perturbed in hMCM7 siRNA-treated cells, whereas this response was clearly blunted in hRad17-depleted cells. The latter results are consistent with the model that ATM, rather than ATR, serves as the primary upstream activator of hChk2 in response to IR-induced DSBs (Bartek and Lukas, 2003; Shiloh, 2003).

During DNA replication, hMCM7 interacts with DNA as one member of the hexameric hMCM2–7 complex (Tye, 1999;

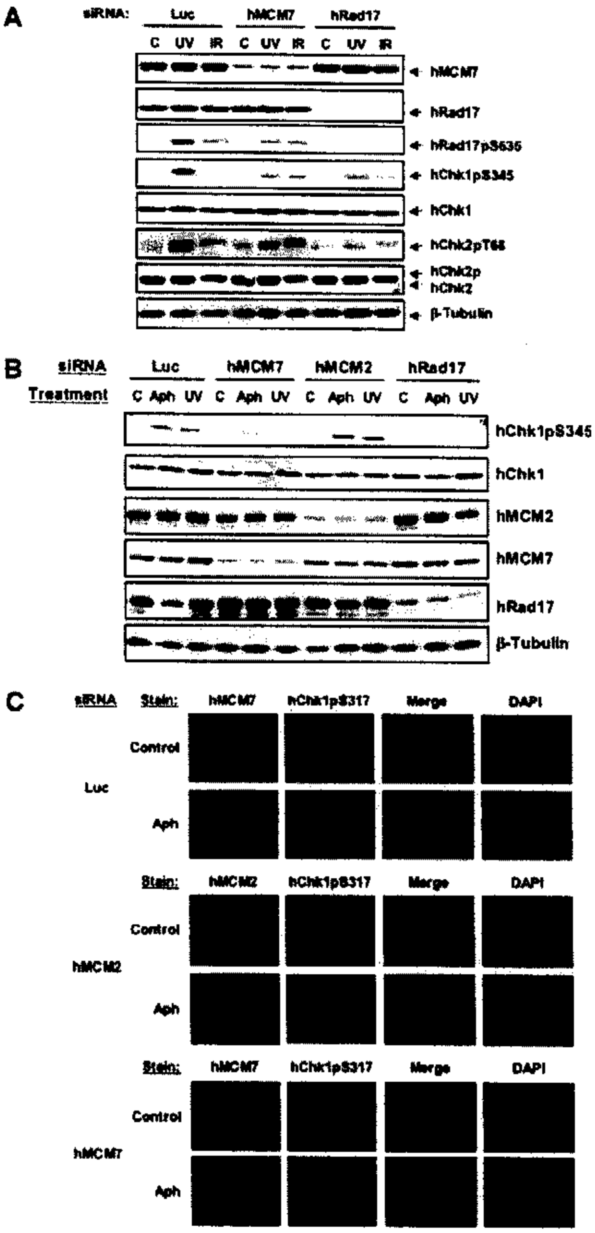

Figure 2 Depletion of hMCM7 and hRad17 inhibits genotoxic stress-induced hChk1 phosphorylation. (**A**) HeLa cells were transfected with hMCM7 or hRad17 siRNA, and then treated with 200 J/m^2 of UV or 20 Gy IR at 48 h post-transfection. Cellular extracts were harvested at 1 h after UV or IR treatment, and cell extracts (50 μg protein) were immunoblotted with the indicated antibodies. β-Tubulin served as a sample-loading control. (**B**) hMCM7, hMCM2, or hRad17 siRNA-transfected HeLa cells were treated for 4 h with 200 J/m^2 UV light or 1 μM Aph, and cellular extracts were separated by SDS–PAGE and immunoblotted with the indicated antibodies. (**C**) Cells were transfected with hMCM7 or hMCM2 siRNA, and were treated with 1 μM Aph. Cells were fixed after 4 h and stained with the indicated antibodies. Cell nuclei were stained with DAPI.

Labib and Diffley, 2001). To determine whether the S-phase checkpoint functions of hMCM7 was uniformly dependent on the MCM2–7 proteins, we silenced hMCM2 gene expression in HeLa cells by transfection with specific siRNA. Exposure to either siRNA reduced expression of the cognate MCM protein by at least 80%, relative to the Luc siRNA-treated control cells. We noted that depletion of hMCM2 caused a moderate reduction of hMCM7 expression and *vice versa*, suggesting that loss of one MCM protein might negatively impact the stability of other members of the hMCM2–7 complex (Figure 2B). Nonetheless, reduced expression of hMCM2 had no effect on genotoxic stress-induced hChk1 phosphorylation, whereas this response was profoundly suppressed in the hMCM7 siRNA-treated cells (Figure 2B and C). Signal relay from ATR to hChk1 was also not perturbed in hMCM4 siRNA-treated cells (not shown). Thus, these observations indicate that the efficiency of ATR signaling to hChk1 is particularly sensitive to reduced expression of the hMCM7 subunit.

We previously reported that UV light-induced DNA damage triggers the appearance of ATR-containing nuclear foci, which represent surrogate markers for ATR activation at sites of DNA damage (Tibbetts *et al*, 2000). In the present study, we determined whether the formation of these ATR nuclear foci was impaired in hMCM7- or hRad17-depleted cells. As shown previously (Tibbetts *et al*, 2000), the majority of nuclei in nonirradiated cells exhibited a diffuse nuclear staining pattern for ATR, which shifted to a punctate staining pattern at 6 h post-UV irradiation (Figure 3A). The formation of ATR nuclear foci was not impaired in hRad17-depleted cells (Figure 3A); indeed, these cells displayed a clear in-

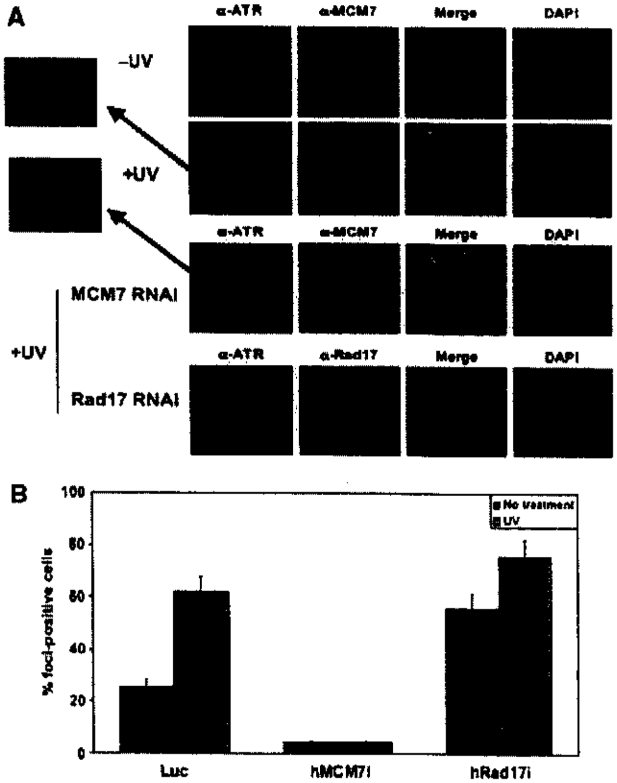

Figure 3 Loss of hMCM7 interferes with the formation ATR nuclear foci in UV-damaged cells. (**A**) Immunofluorescence microscopy. HeLa cells were either left untreated or irradiated with 200 J/m^2 UV light. Cells were fixed after 6 h, and immunostained with α-ATR or α-hMCM7 antibodies. In the bottom two rows, the cells were transfected with the indicated siRNAs, and were irradiated with UV light after 48 h. DAPI staining was used to mark cell nuclei. (**B**) Quantitation of ATR nuclear foci in the cell populations shown in panel A. Triplicate samples (minimum, 100 cells per sample) were counted manually by microscopy. Error bars indicate standard error of the mean from the three sample populations.

crease in the number of nuclear foci-positive cells in the absence of UV exposure (Figure 3B). The latter results suggest that knockdown of hRad17 expression triggers the accumulation of spontaneous, unrepaired DNA damage in cycling HeLa cells. The consequences of hMCM7 protein depletion were strikingly different, as the appearance of ATR nuclear foci was virtually abolished in both nonirradiated and UV-irradiated cells (Figure 3A and B). Thus, hMCM7 expression appears to be critical for the recruitment of ATR into nuclear foci following cellular exposure to certain types of genotoxic stress.

Under normal conditions, recognition of DNA damage during S phase slows replication fork progression and inhibits the firing of late origins of replication (Lopes *et al*, 2001; Tercero and Diffley, 2001). We predicted that impairment of the UV-induced DNA damage response might yield a UV-resistant DNA synthesis (UVDS) phenotype, which is analogous to the radio-resistant DNA synthesis (RDS) defect associated with loss of ATM and other proteins involved in the checkpoint response to DNA DSBs incurred during S phase (Painter and Young, 1980). We found that hMCM7 siRNA-treated A549 cells displayed elevated levels of DNA synthesis at 30–120 min after UV light exposure (Figure 4A and B), consistent with the induction of UVDS. In contrast, these cells did not exhibit the RDS defect after IR exposure, indicating that hMCM7 expression was specifically required for S-phase checkpoint activation in response to UV-induced DNA damage. As expected, given the more global role of hRad17 in DNA damage signaling, transfection of the cells with hRad17 siRNA evoked both UVDS and RDS (Figure 4B and C).

Based on the above-noted defects in ATR function in hMCM7-depleted cells, we predicted that these cells might fail to delay S-phase progression in response to UV-induced DNA damage. However, testing of this prediction was complicated by the possibility that reduced hMCM7 might lead to an intrinsic defect in S-phase progression. Consequently, we examined S- to M-phase progression in hMCM7 siRNA-treated HeLa cells with a nocodazole capture assay. Flow cytometric analyses of propidium iodide (PI)-stained cells revealed that both hRad17- and hMCM7-depleted cells accumulated with 4*N* DNA content in the presence of nocodazole, indicating that these cell populations had successfully replicated their DNA (Supplementary Figure S1A). To monitor more accurately cell-cycle progression in hMCM7 siRNA-treated cells, we pulse-labeled these cells with BrdU and monitored the cell-cycle status of the labeled cells by PI staining over the following 24 h. Relative to the Luc siRNA-treated control cells, BrdU-positive cells depleted of hMCM2, hMCM4 (not shown), or hMCM7 displayed little or no defect in S-phase progression (Supplementary Figure S1B and C). In subsequent experiments, we transfected HeLa cells with siRNAs targeted against green fluorescent protein (GFP), hMCM7, or hRad17, and treated the cells with 200 J/m^2 UV and examined their cell-cycle distributions at 24 h postirradiation. In GFP siRNA-transfected cells, UV irradiation provoked an increase in the percentage of S-phase cells after 24 h, consistent with a checkpoint-mediated delay of cell-cycle progression through S phase (Figure 5A). In contrast, hMCM7 siRNA-treated cells arrested predominantly in G$_2$/M phase after UV light exposure. These results indicate that impaired S-phase checkpoint activation in the hMCM7-depleted cells favors the progression of cells bearing damaged DNA into G$_2$ phase, where they are captured by the G$_2$ DNA damage checkpoint.

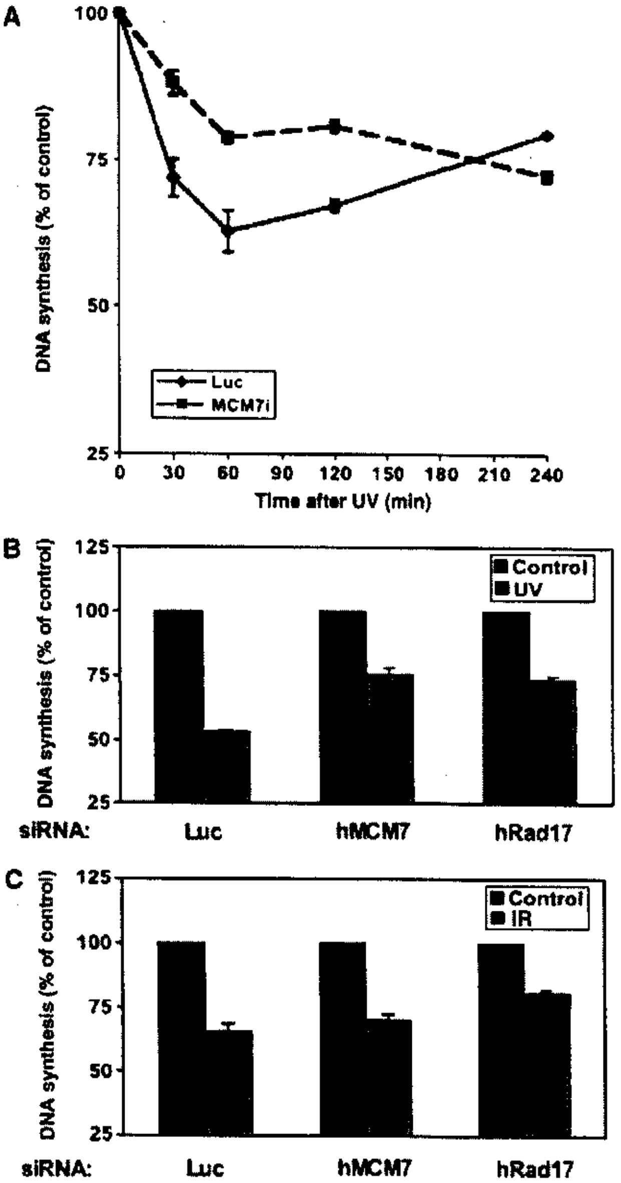

Figure 4 hMCM7 depletion confers a UVDS phenotype. (**A**) Time course of UVDS. A549 cells were treated with the indicated siRNAs, and, after 48 h, were irradiated with 200 J/m^2 UV light. DNA synthesis was measured at the indicated times after UV light exposure as described in Materials and methods. Error bars indicate standard deviation from quadruplicate samples. (**B**) Effect of hMCM7 versus hRad17 siRNA treatment on UVDS. A549 cells were treated with the indicated siRNAs, and were irradiated with 200 J/m^2 UV light at 48 h post-transfection. DNA synthesis was determined at 1 h after radiation exposure. (**C**) Effect of hMCM7 versus or hRad17 depletion on RDS. A549 cells were treated with the indicated siRNAs, and were irradiated with 20 Gy IR at 48 h post-transfection. DNA synthesis was determined at 1 h after radiation exposure.

Cell-cycle checkpoint malfunctions are frequently associated with heightened sensitivity to killing by DNA-damaging agents. Treatment of hMCM7- or hRad17-depleted HeLa cells with UV light revealed that both of these cell populations were sensitized to the antiproliferative and/or cell-killing

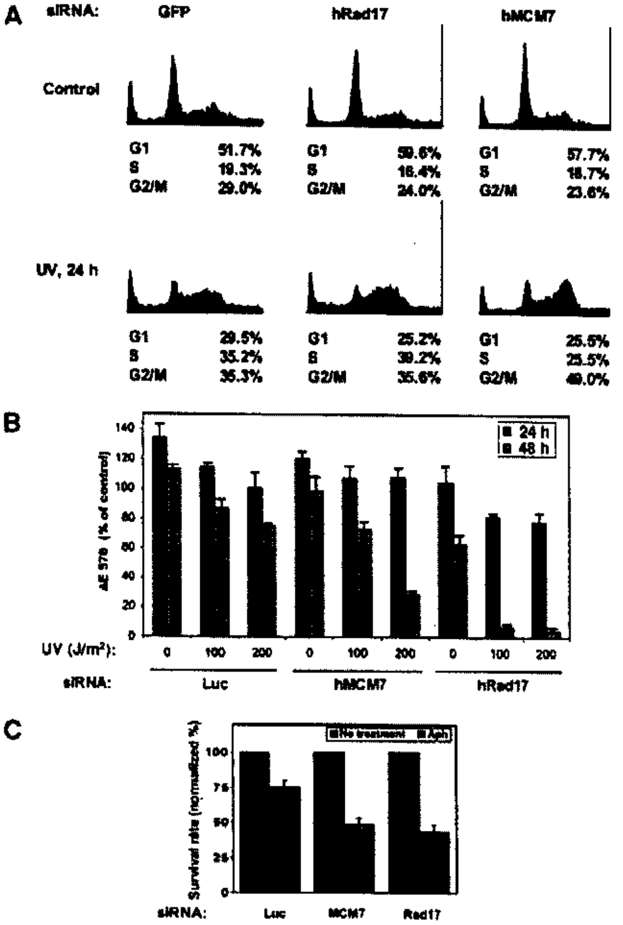

Figure 5 Effects of hMCM7 or hRad17 depletion on UV-induced S-phase checkpoint activation and cell survival. (**A**) Impaired S-phase checkpoint in hMCM7-depleted cells. HeLa cells were transfected with the indicated siRNAs, and then exposed to 200 J/m^2 UV light at 48 h after transfection. Cell-cycle distributions were examined at 24 h postirradiation. (**B**) Cell survival after UV irradiation. HeLa cells were transfected with indicated siRNA and, after 48 h, were exposed to the indicated doses of UV light. Cell survival was determined after 24 or 48 h in triplicate samples with an MTT dye conversion assay. Absorbance values were normalized to the zero time control for each cell population, which was arbitrarily set at 100%. Error bars indicate standard deviations. (**C**) Effect of hMCM7 or hRad17 depletion on cellular recovery from an Aph block. HeLa cells were transfected with the indicated siRNAs, and were replated after 24 h for survival assays. After 24 h in culture, the cells were treated for 16 h with 1 μM Aph, and then released into drug-free medium. Cell survival was determined in quadruplicate samples as described in Materials and methods. Values were normalized to the untreated control for each cell population, and error bars indicate standard deviations.

activities of UV light, relative to that observed in the Luc siRNA-treated control cells (Figure 5B). Finally, we examined the clonogenic survival of hMCM7- or hRad17-depleted cells after transient DNA replication stress imposed by Aph. Once again, recovery of the cells after release from Aph-induced replication arrest was impaired in the hMCM7 and hRad17 siRNA-treated cell populations (Figure 5C).

Mapping studies of the hRad17–hMCM7 interaction with the yeast two-hybrid system revealed that a 43-amino-acid peptide fragment (designated MCM7-5) from hMCM7 was sufficient to bind detectably to full-length hRad17 (Supplementary Figure S2). Conversely, the minimal hRad17-derived peptide capable of binding to an amino-terminally truncated hMCM7 bait protein (fragment designated R27 in the hMCM7 deletion series) encompassed 249 amino acids from the carboxyl terminus of hRad17 (indicated as Rad17-5 in Supplementary Figure S2). We constructed GFP fusion proteins containing the MCM7-5 or Rad17-5 fragments with the idea in mind that these fusion proteins, when ectopically expressed in human cells, might interfere with the physical and functional interactions between endogenous hMCM7 and hRad17.

In preliminary studies, we found that transient expression of a GFP-tagged fusion protein containing the MCM7-5 fragment completely blocked S-phase entry and progression in otherwise unperturbed HeLa cells (results not shown). In contrast, HeLa cells transfected with the GFP-Rad17-5 construct continued to cycle normally, a finding that prompted us to focus on this fusion protein as a potential inhibitor of signal relay between hMCM7 and hRad17 in UV light- or Aph-stressed cells. Co-immunoprecipitation experiments revealed that endogenous hMCM7 associated with the ectopically expressed GFP-Rad17-5 fusion protein in transfected HeLa cells (Figure 6A). In contrast, α-hMCM7 immunoprecipitates from GFP-transfected cells contained no detectable GFP, in spite of the fact that intact GFP was expressed at much higher levels than the GFP-Rad17-5 fusion protein. Furthermore, GFP-Rad17-5 expression reduced the level of endogenous hRad17 present in α-hMCM7 immunoprecipitates (Figure 6A), consistent with the idea that the Rad17-5 fragment competitively interferes with the hMCM7–hRad17 interaction in intact cells. In subsequent studies, we found that expression of GFP-Rad17-5 strongly suppressed the phosphorylation of hChk1 in UV- and Aph-treated cells (Figure 6B). Furthermore, like their hMCM7-depleted counterparts, the GFP-Rad17-5-expressing HeLa cells displayed the UVDS phenotype (Figure 6C).

To rule out the possibility that the GFP-Rad17-5 construct was simply acting as a dominant suppressor of hRad17 function, we determined whether the GFP-Rad17-5-transfected cells retained the ability to phosphorylate hChk2 after IR exposure. This ATM-dependent response also hinges on the function of hRad17 (see Figure 2A). Indeed, the GFP-Rad17-5-transfected cells displayed a strong increase in basal hChk2 phosphorylation, which was modestly increased by IR or UV exposure, while hChk1 activation was virtually abolished (Figure 6D). The basal increase in hChk2 phosphorylation in GFP-Rad17-5-expressing cells is consistent with the observation that these cells suffer spontaneous DNA damage (see Figure 7A), possibly due to disruption of the replication checkpoint. Regardless, we conclude from these results that the GFP-Rad17-5 construct is a selective, rather than global, inhibitor of hRad17 functions in radiation-damaged cells.

ATR-deficient cells rapidly accumulate damaged DNA in the absence of extrinsic genotoxic stress, likely due to a breakdown in the fidelity of DNA replication (Brown and Baltimore, 2000; de Klein *et al*, 2000). Given the evidence that GFP-Rad17-5 expression disrupts signal transmission through ATR to hChk1, we predicted that cells transfected with this construct might also display elevated levels of spontaneous DNA damage. Consequently, we stained the cells with a phosphospecific antibody that recognizes an ATM/ATR-dependent phosphorylation site (Ser-139) in histone H2AX. The phosphorylated form of H2AX (γH2AX) accumulates rapidly at sites of DNA damage in cells treated with IR, UV, or DNA replication inhibitors (Rogakou *et al*, 1998, 1999; Ward and Chen, 2001). Expression of GFP-Rad17-5 in HeLa cells provoked a greater than seven-fold increase in γH2AX-positive nuclei, compared with the GFP-transfected control cells (Figure 7A and B). We repeated these experiments with hMCM7 siRNA-treated cells, and observed that loss of hMCM7 also caused a clear increase in γ-H2AX-positive nuclei (Figure 7C). In contrast, no increase in γH2AX staining was seen in the hRad17-depleted cells. The latter results suggest that loss of hRad17 does not lead to spontaneous DNA damage, although we have not ruled out the possibility that hRad17 expression is required for maximal phosphorylation of histone H2AX in UV-damaged cells.

Discussion

The hexameric MCM complex plays central roles in DNA replication initiation and elongation (Labib *et al*, 2000). In this study, we have defined an additional role for the hMCM7 subunit of the MCM complex in the activation of the S-phase DNA damage checkpoint by UV light and Aph. Our results argue that hMCM7 participates in functionally important interactions with at least two key elements of the S-phase checkpoint machinery—hRad17 and ATRIP-ATR. Manipulations that interfere with hMCM7 function strongly inhibit the formation of ATR nuclear foci, as well as ATR-dependent hChk1 activation in UV- or Aph-stressed cells. Furthermore, disruption of hMCM7 function triggered a DNA damage response in the absence of extrinsic genotoxic stress, consistent with previous evidence that the ATR–Chk1 pathway plays a continuous role in genome maintenance during normal DNA replication (Casper *et al*, 2002; Cha and Kleckner, 2002; Marheineke and Hyrien, 2004). Taken together, these results strengthen the notion that components of the DNA replication fork function as both DNA damage sensors and effectors of checkpoint signaling in S-phase cells (Tercero *et al*, 2003).

Previous studies identified at least three binding partners for hRad17: Ku-interacting protein (Chang *et al*, 1999), hRad1 (Rauen *et al*, 2000), and protein phosphatase 5 (Ali *et al*, 2004). With the exception of the hRad1 interaction, the functional significance of these associations with hRad17 remains unclear. Our results indicate that binding of hRad17 to hMCM7 is critical for S-phase checkpoint signaling in response to replicative stress. Studies in fission yeast have shown that Rad17 is constitutively bound to chromatin, and that the level of chromatin-bound Rad17 is increased during replication stress (Kai *et al*, 2001). More recent results indicate that the hRad17–RFC2–5 complex binds most avidly to DNA duplexes containing single-stranded gaps coated with RPA (Zou *et al*, 2003). These structures are abundant in the vicinity of active replication forks (Waga and Stillman, 1998). In light of the current findings, we propose that RPA recruits

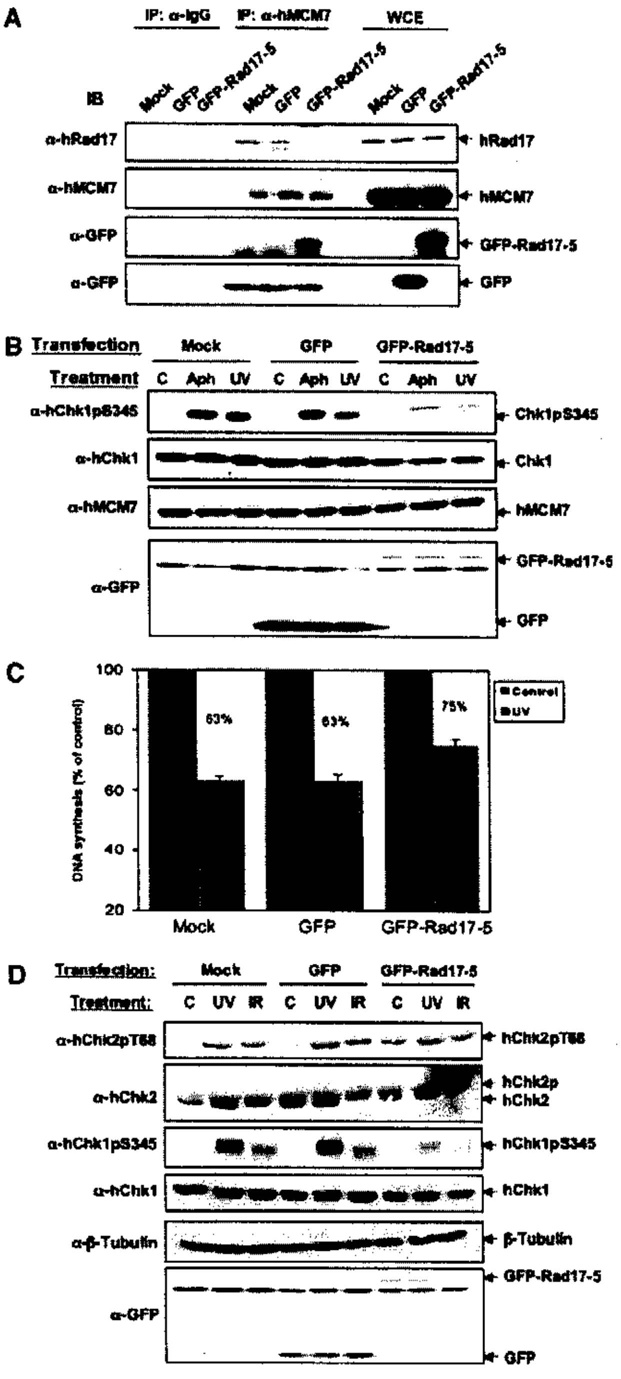

Figure 6 Disruption of S-phase checkpoint signaling by ectopic expression of the hMCM7-binding region of hRad17. (**A**) Binding of ectopically expressed GFP-Rad17-5 to endogenous hMCM7. HeLa cells were transfected with GFP or GFP-Rad17-5 fusion construct (encoding amino acids 421–670 of hRad17). At 24 h post-transfection, the cells were harvested, lysed, and detergent-soluble protein (1 mg) was immunoprecipitated with α-hMCM7 antibody. (**B**) hChk1 phosphorylation. HeLa cells were transfected with GFP or GFP-Rad17-5, and then treated with 200 J/m^2 UV light or 1 μM Aph at 16 h after transfection. Cellular extracts (50 μg protein) were prepared after 2 h, and proteins were immunoblotted with the indicated antibodies. (**C**) Ectopically expressed GFP-Rad17-5 confers a UVDS phenotype. HeLa cells were transiently transfected with either GFP- or GFP-Rad17-5-encoding plasmids, and, after 24 h, were irradiated with 200 J/m^2 UV light. DNA synthesis was determined at 1 h after radiation exposure as described in Materials and methods. (**D**) hChk2 phosphorylation. HeLa cells were transfected with GFP or GFP-Rad17-5, and then treated with 200 J/m^2 UV light or 20 Gy of IR at 16 h after transfection. Cellular extracts (50 μg protein) were prepared after 2 h, and proteins were immunoblotted with the indicated antibodies.

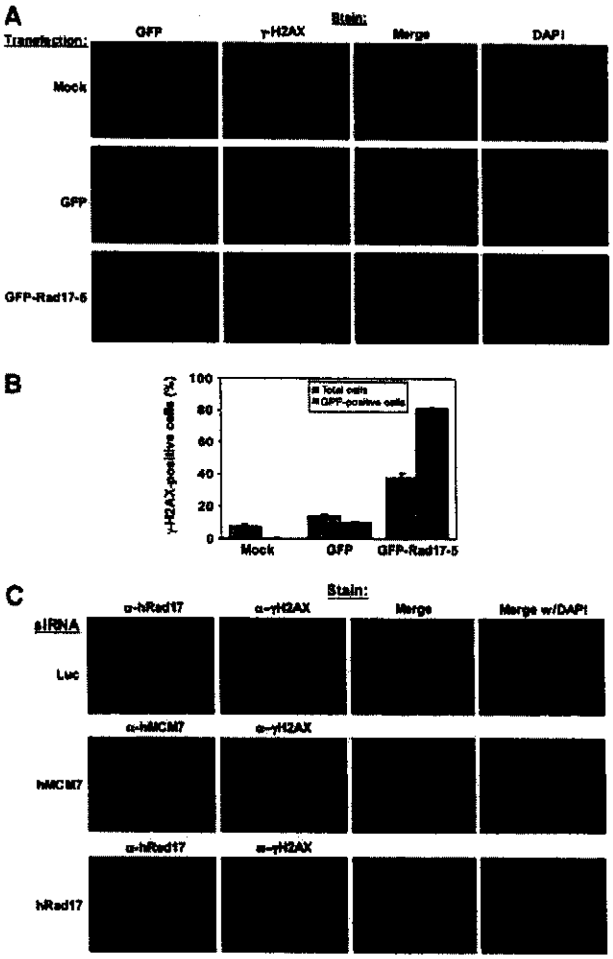

Figure 7 Histone H2AX phosphorylation. (**A**) γH2AX staining. HeLa cells were transiently transfected with either GFP- or GFP-Rad17-5-encoding plasmids, then were fixed and permeabilized after 16 h in culture. The cells were stained with γH2AX-specific antibody (red), and cell nuclei were counterstained with DAPI (blue). (**B**) Quantitation of γH2AX-positive cell nuclei. A minimum of 100 cells from each of the cell populations depicted in panel A were scored for nuclear γH2AX staining. (**C**) γH2AX staining in hMCM7- versus hRad17-depleted HeLa cells. The indicated siRNA-transfected cells were costained with α-γH2AX antibody (red) and either hRad17 or hMCM7 antibody (green) after 60 h in culture. Cell nuclei were counterstained with DAPI (blue).

the hRad17–RFC2–5 complex to sites of DNA replication, where hRad17 encounters and binds to hMCM7 in the vicinity of the replication fork. We further speculate that replication fork stalling induced by agents such as UV or Aph triggers a local increase in the level of RPA-bound ssDNA, an event that provokes the recruitment of sufficient hRad17–RFC2–5, together with ATRIP-ATR, to elicit a full-blown S-phase damage checkpoint response. This model is consistent with the notion that DNA polymerase stalling induced by Aph causes DNA helicase activity to become uncoupled from DNA polymerases in the *Xenopus* egg extracts, an event that would lead to abnormally long stretches of ssDNA (Walter and Newport, 2000).

Although the present study has focused exclusively on hMCM7, this protein is generally believed to function in the context of the hMCM2–7 complex during DNA replication. Remarkably, however, depletion of either hMCM4 or hMCM2 failed to disrupt the activation of hChk1 by UV or Aph, indicating that the checkpoint signaling function of hMCM7 may not be obligatorily linked to its role in DNA replication. A recent report provides strong support for the idea that MCM proteins are functionally intertwined with the S-phase checkpoint machinery (Cortez *et al*, 2004). This study demonstrated that two MCM proteins, hMCM2 and hMCM3, are phosphorylated by ATR and ATM, respectively, in cells exposed to genotoxic stress. These findings complement an earlier study, which showed that hMCM4 also undergoes rapid phosphorylation in cells exposed to UV light or DNA replication inhibitors (Ishimi *et al*, 2003). Although the functional significance of these phosphorylation events is unknown, it seems clear that, as is the case for ATR and hRad17, the interplay between ATR and the MCM proteins will prove to be bidirectional. Cortez and Elledge also found that hMCM7 was a direct binding partner for the ATR-associated protein ATRIP, and showed that partial depletion of hMCM7 interfered with UV-induced hChk1 activation in U2OS cells. Taken together with our findings, these results provide compelling evidence to support the hypothesis that hMCM7 plays a specialized role in the relay of DNA damage signals from the replication fork to proximal elements of the S-phase checkpoint pathway.

Costanzo *et al* (2003) have defined a distinct mechanism of S-phase checkpoint initiation in *Xenopus* egg extracts (Costanzo *et al*, 2003). These investigators demonstrated that the Dbf4–Cdc7 kinase was a critical target of the ATR-dependent S-phase checkpoint induced by the topoisomerase II inhibitor etoposide. Inhibition of Dbf4–Cdc7 by this checkpoint mechanism interferes with the phosphorylation of the MCM complex by this protein kinase, thereby suppressing conversion of pre-RCs to active replication forks. The inhibition of replication origin firing via this checkpoint mechanism complements the pathway outlined in the present study, which relies on hMCM7 function to slow the progress of and/or stabilize pre-existing replication forks that encounter fork-stalling lesions. The common element that leads to the recruitment of ATR in both of these S-phase checkpoint pathways is the formation of RPA-coated ssDNA (Costanzo *et al*, 2003; Zou and Elledge, 2003; Zou *et al*, 2003).

Our results indicate that hRad17 and hMCM7 interact in a pathway that includes ATR and mediates S-phase checkpoint activation in response to genotoxic stress. However, it is clear that binding to hMCM7 is not the only mechanism by which hRad17 receives and transmits DNA damage signals in human cells. For example, we observed that UV- or IR-induced hChk2 phosphorylation was not impaired in hMCM7-deficient cells, whereas this event was strongly suppressed in cells depleted of hRad17. This outcome is consistent with previous evidence that hChk2 phosphorylation and activation are more tightly coupled to ATM than to ATR (Matsuoka *et al*, 2000; Bartek and Lukas, 2003). The checkpoint functions of hRad17 are not confined to S phase (Elledge, 1996; Caspari and Carr, 1999), suggesting that, at other points in the cell cycle, DNA damage signals are relayed to hRad17 through RPA and hRad17-interacting proteins other than hMCM7.

A striking outcome of our studies was that decreased hMCM7 expression abolished the formation of ATR-containing nuclear foci in UV-treated cells. In contrast, depletion of hRad17 actually promoted the formation of such foci in the absence of genotoxic agents. These observations reinforce the idea that the ATRIP-ATR and 9-1-1 complexes are recruited independently to damaged chromatin (Zou *et al*, 2002). While RPA may be required for efficient recruitment of ATRIP-ATR to ssDNA (Zou and Elledge, 2003), the present findings, together with those of Cortez *et al* (2004), suggest that hMCM7 is centrally involved in the assembly and/or stabilization of ATRIP-ATR-containing foci in S-phase cells. In line with this model, we found that α-ATRIP immunoprecipitates from cycling cells contain both hMCM7 and hRad17, and that the amounts of co-precipitating hMCM7 and hRad17 are increased after cellular exposure to UV or Aph (Figure 1B).

In summary, the present findings highlight the hMCM7 protein as a critical node for checkpoint signal transmission from stalled replication forks. A major unresolved issue is whether the checkpoint signaling functions of hMCM7 are strictly tied to its role in DNA replication as part of the MCM complex, or whether this facet of hMCM7 function is independent of its contributions to normal DNA replication. Regardless, the present studies add significant strength to the idea that the replication fork functions as both a sensor of DNA damage and an effector of checkpoint signaling in cells that incur DNA damage during S phase (Cimprich, 2003; Katou *et al*, 2003; Tercero *et al*, 2003; Oehlmann *et al*, 2004).

Materials and methods

Cell culture and antisera

U2OS osteosarcoma and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). A549 lung carcinoma cells were grown in high-glucose DMEM/F12 supplemented with 10% FBS. Polyclonal antibodies against ATRIP were prepared by immunizing rabbits with a glutathione S-transferase fusion protein containing amino acids 1–181 from human ATRIP. Polyclonal antibodies against hMCM2 were kindly provided by Dr Wei Jiang (The Burnham Institute). Commercial antibodies were obtained from the following sources (in parentheses): α-hMCM4 (Abcam); α-hMCM7 (Sigma); α-hRad17 and α-hChk1 (Santa Cruz); α-phospho-hChk1 (pSer-317 and pSer-345) and α-Chk2 (Cell Signaling); α-ATR (Affinity Bioreagents); α-γH2AX (Cell Signaling); monoclonal α-FLAG M2 (Sigma); Alexa488-conjugated monoclonal α-BrdU (Molecular Probes); α-HA, clone 12CA5 (BabCo), α-phospho-Chk2 (pT-68) (R&D systems). Aph was prepared as a stock solution in dimethylsulfoxide and stored at −80°C until use.

Plasmids and constructs

Plasmid expression vectors for HA-tagged mouse MCM7 and FLAG-tagged human hRad17 were constructed in pcDNA3.1 (Invitrogen). For yeast two-hybrid screening, fragments of hRad17 and hMCM7 were subcloned into pBridge and pACT2 vectors (Clontech), respectively. The interacting fragments of hRad17 and hMCM7 were subcloned into pEGFP-C2 (Clontech). U2OS cells were transfected with Fugene 6 (Roche) in accordance with the manufacturer's suggested protocol. HeLa cells were transfected with plasmid DNAs with Lipofectamine 2000 (Invitrogen).

siRNA transfections

The siRNA duplexes targeted against hMCM7, hMCM2, hMCM4, hRad17, GFP, and Luc were purchased from Dharmacon Research Inc. (Lafayette, CO). The hRad17 siRNA target sequence was designed as previously reported (Zou *et al*, 2002) and the target sequences for the hMCM2, hMCM4, and hMCM7 siRNAs are TCATCGGAATCCTTCACCA, TGTTACAGGCATCTATCGA, and CTCGGGAAGAAGCAGTTCA, respectively. Cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen) according to the manufacturer's suggested protocol.

Cell-cycle analysis

For fluorescence-activated cell sorting (FACS) analysis, cells were ethanol-fixed, washed in phosphate-buffered saline (PBS), and were stained for 30 min at 37°C with 10 μg/ml RNase A and 20 μg/ml PI. For PI staining of GFP-expressing cells, the cells were first fixed for 10 min in 0.5% paraformaldehyde on ice, washed twice in PBS, and stained as described above. Cells were analyzed with a flow cytometer (FACSort, BD Sciences) and CellQuest Pro software.

Yeast two-hybrid screen

The full-length human hRad17 cDNA was subcloned into a yeast multicopy plasmid carrying the GAL4-binding domain (pBridge, Clontech, La Jolla, CA). The construct was then transformed into yeast strain A109. The transformants were selected, and then secondarily transformed with a human fetal brain cDNA library constructed in pACT2 vector (Clontech). Transformants were screened for growth on plates lacking adenine, tryptophan, histidine, and leucine. After 3–6 days at 30°C, approximately 10^6 colonies were obtained, and positive clones were further examined for α-galactosidase expression. Plasmids harboring interacting cDNAs were rescued from positive yeast colonies, transformed into *Escherichia coli* KC8 strain, and positive transformants selected for growth on leucine-deficient M9 medium. The interacting cDNAs were characterized by nucleotide sequencing.

Co-immunoprecipitation

Cells were harvested by trypsinization and centrifugation, and then were resuspended in lysis buffer (150 mM sodium chloride, 5 mM β-glycerophosphate, 5 mM EDTA, pH 7.4, containing 1 mM DTT and 1% NP-40, supplemented with 10 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin. After 15 min on ice, the lysate was centrifuged for 5 min at 9000 *g*, and cleared extracts were immunoprecipitated with 2 μg of primary antibody. Depending on the type of primary antibody, either mouse monoclonal immunoglobulin G (IgG) or polyclonal rabbit α-mouse IgG was used as a negative control. Immune complexes were precipitated for 2 h at 4°C with either protein A–Sepharose for polyclonal antibodies or anti-mouse IgG–Sepharose for monoclonal antibodies. Immunoprecipitates were washed three times with lysis buffer, resuspended in 40 μl 2 × SDS–PAGE sample buffer, and samples were heated for 5 min at 100°C. Soluble proteins were resolved by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with the indicated antibodies.

UV-resistant DNA synthesis assay

A549 cells were labeled for 24 h with 20 nCi/ml methyl-^{14}C-thymidine (Amersham) at the time of siRNA transfection. The medium was removed and replaced with fresh medium. The ^{14}C-labeled cells were then treated with the indicated doses of UV-B light. After 1 h, the cells were pulsed for 40 min with 2 mCi/ml methyl-^{3}H-thymidine (Amersham). The doubly labeled cells were washed with PBS, fixed in 80% ethanol, and stored at −20°C for at least 3 h. The cells were then washed two times with 80% ethanol. NaOH (0.5 ml, 0.25 M) was added to each cell pellet, and incorporation of ^{14}C and ^{3}H into DNA was measured by dual-isotope liquid scintillation counting. The ratio of ^{3}H-labeled DNA to ^{14}C-labeled DNA was determined for each sample, and normalized to the ratio obtained in the nonirradiated control samples.

Immunofluorescence microscopy

HeLa cells were grown on glass coverslips coated with poly-L-lysine (Sigma). At the indicated time points, the cells were washed twice with PBS, and were fixed for 10 min in PBS containing 3.75% formaldehyde and 0.2 M sucrose. Samples were rehydrated in PBS, and incubated for 30 min in blocking solution (PBS containing 10% FBS and 0.4% Triton X-100). Samples were subsequently overlaid for 1 h with primary antibodies at room temperature. After three washes in PBS and 0.1% Triton X-100, the samples were overlaid for 1 h with Texas red-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG diluted 1:500 in PBS and 0.1% Triton X-100. Samples were then washed and incubated for 5 min in 1 μg/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) to stain nuclear DNA. After extensive washing, the specimens were mounted with coverslips and an aqueous anti-fade mounting reagent (Vectashield, Vector Laboratories).

Cell proliferation and clonogenic assays

Effects of UV on cell proliferation and viability were determined with an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, HeLa cells were seeded into 12-well dishes, transfected with indicated siRNA, and then UV irradiated at 48 h after transfection. Samples were prepared by removal of the culture medium and addition of 250 µl of fresh medium containing 0.5 mg/ml MTT. After 2 h at 37°C, the cells were lysed with 250 µl 2-butanol/2-propanol/1 N HCl (16/8/1, v/v/v). The soluble material was transferred to flat-bottomed 96-well plates and the absorbance at 570 nm was determined with an automated late reader.

For clonogenic assay, HeLa cells were transfected with siRNAs, and, after 24 h, the cells were replated in 60-mm dishes and treated as described in the figure legend. Colonies were stained with crystal violet, and dye-bound protein was solubilized at 37°C with 0.5% SDS in 50% ethanol. Samples were analyzed by absorbance spectroscopy at 590 nm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Abraham RT (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* **15**: 2177–2196

Ali A, Zhang J, Bao S, Liu I, Otterness D, Dean NM, Abraham RT, Wang XF (2004) Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. *Genes Dev* **18**: 249–254

Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**: 499–506

Bao S, Tibbetts RS, Brumbaugh KM, Fang Y, Richardson DA, Ali A, Chen SM, Abraham RT, Wang XF (2001) ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. *Nature* **411**: 969–974

Bartek J, Lukas J (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3**: 421–429

Bermudez VP, Lindsey-Boltz LA, Cesare AJ, Maniwa Y, Griffith JD, Hurwitz J, Sancar A (2003) Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex *in vitro*. *Proc Natl Acad Sci USA* **100**: 1633–1638

Brown EJ, Baltimore D (2000) ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* **14**: 397–402

Burtelow MA, Kaufmann SH, Karnitz LM (2000) Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. *J Biol Chem* **275**: 26343–26348

Carson CT, Schwartz RA, Stracker TH, Lilley CE, Lee DV, Weitzman MD (2003) The Mre11 complex is required for ATM activation and the G2/M checkpoint. *EMBO J* **22**: 6610–6620

Caspari T, Carr AM (1999) DNA structure checkpoint pathways in *Schizosaccharomyces pombe*. *Biochimie* **81**: 173–181

Casper AM, Nghiem P, Arlt MF, Glover TW (2002) ATR regulates fragile site stability. *Cell* **111**: 779–789

Cha RS, Kleckner N (2002) ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. *Science* **297**: 602–606

Chang MS, Sasaki H, Campbell MS, Kraeft SK, Sutherland R, Yang CY, Liu Y, Auclair D, Hao L, Sonoda H, Ferland LH, Chen LB (1999) HRad17 colocalizes with NHP2L1 in the nucleolus and redistributes after UV irradiation. *J Biol Chem* **274**: 36544–36549

Cimprich KA (2003) Fragile sites: breaking up over a slowdown. *Curr Biol* **13**: R231–R233

Cortez D, Glick G, Elledge SJ (2004) Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci USA* **101**: 10078–10083

Cortez D, Guntuku S, Qin J, Elledge SJ (2002) ATR and ATRIP: partners in checkpoint signaling. *Science* **294**: 1713–1716

Costanzo V, Shechter D, Lupardus PJ, Cimprich KA, Gottesman M, Gautier J (2003) An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. *Mol Cell* **11**: 203–213

de Klein A, Muijtjens M, van Os R, Verhoeven Y, Smit B, Carr AM, Lehmann AR, Hoeijmakers JH (2000) Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. *Curr Biol* **10**: 479–482

Elledge SJ (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* **274**: 1664–1672

Heffernan TP, Simpson DA, Frank AR, Heinloth AN, Paules RS, Cordeiro-Stone M, Kaufmann WK (2002) An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol Cell Biol* **22**: 8552–8561

Hekmat-Nejad M, You ZS, Yee MC, Newport JW, Cimprich KA (2000) *Xenopus* ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint. *Curr Biol* **10**: 1565–1573

Ishimi Y, Komamura-Kohno Y, Kwon HJ, Yamada K, Nakanishi M (2003) Identification of MCM4 as a target of the DNA replication block checkpoint system. *J Biol Chem* **278**: 24644–24650

Jiang KC, Pereira E, Maxfield M, Russell B, Goudelock DM, Sanchez Y (2003) Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345. *J Biol Chem* **278**: 25207–25217

Kai M, Tanaka H, Wang TSF (2001) Fission yeast Rad17 associates with chromatin in response to aberrant genomic structures. *Mol Cell Biol* **21**: 3289–3301

Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* **424**: 1078–1083

Kondo T, Wakayama T, Naiki T, Matsumoto K, Sugimoto K (2001) Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. *Science* **294**: 867–870

Labib K, Diffley JF (2001) Is the MCM2–7 complex the eukaryotic DNA replication fork helicase? *Curr Opin Genet Dev* **11**: 64–70

Labib K, Tercero JA, Diffley JF (2000) Uninterrupted MCM2–7 function required for DNA replication fork progression. *Science* **288**: 1643–1647

Lee JH, Paull TT (2004) Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* **304**: 93–96

Lei M, Tye BK (2001) Initiating DNA synthesis: from recruiting to activating the MCM complex. *J Cell Sci* **114**: 1447–1454

Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* **14**: 1448–1459

Lopes M, Cotta-Ramusino C, Pellicioli A, Liberi G, Plevani P, Muzi-Falconi M, Newlon CS, Foiani M (2001) The DNA replication checkpoint response stabilizes stalled replication forks. *Nature* **412**: 557–561

Lupardus PJ, Byun T, Yee MC, Hekmat-Nejad M, Cimprich KA (2002) A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. *Genes Dev* **16**: 2327–2332

Marheineke K, Hyrien O (2004) Control of replication origin density and firing time in *Xenopus* egg extracts: role of a caffeine-sensitive, ATR-dependent checkpoint. *J Biol Chem* **279**: 28071–28081

Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ (2000) Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* **97**: 10389–10394

Melo JA, Cohen J, Toczyski DP (2001) Two checkpoint complexes are independently recruited to sites of DNA damage *in vivo*. *Genes Dev* **15**: 2809–2821

Michael WM, Ott R, Fanning E, Newport J (2000) Activation of the DNA replication checkpoint through RNA synthesis by primase. *Science* **289**: 2133–2137

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63

Oehlmann M, Score AJ, Blow JJ (2004) The role of Cdc6 in ensuring complete genome licensing and S phase checkpoint activation. *J Cell Biol* **165**: 181–190

Osborn AJ, Elledge SJ (2003) Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. *Genes Dev* **17**: 1755–1767

Painter RB, Young BR (1980) Radiosensitivity in ataxia-telangiectasia: a new explanation. *Proc Natl Acad Sci USA* **77**: 7315–7317

Rauen M, Burtelow MA, Dufault VM, Karnitz LM (2000) The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9. *J Biol Chem* **275**: 29767–29771

Rogakou EP, Boon C, Redon C, Bonner WM (1999) Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J Cell Biol* **146**: 905–916

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* **273**: 5858–5868

Shechter D, Costanzo V, Gautier J (2004) ATR and ATM regulate the timing of DNA replication origin firing. *Nat Cell Biol* **6**: 648–655

Shiloh Y (2003) ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* **3**: 155–168

Stokes MP, Van Hatten R, Lindsay HD, Michael WM (2002) DNA replication is required for the checkpoint response to damaged DNA in *Xenopus* egg extracts. *J Cell Biol* **158**: 863–872

Storchova Z, Pellman D (2004) From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* **5**: 45–54

Tercero JA, Diffley JF (2001) Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature* **412**: 553–557

Tercero JA, Longhese MP, Diffley JF (2003) A central role for DNA replication forks in checkpoint activation and response. *Mol Cell* **11**: 1323–1336

Tibbetts RS, Cortez D, Brumbaugh KM, Scully R, Livingston D, Elledge SJ, Abraham RT (2000) Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. *Genes Dev* **14**: 2989–3002

Tye BK (1999) MCM proteins in DNA replication. *Annu Rev Biochem* **68**: 649–686

Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y (2003) Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* **22**: 5612–5621

Waga S, Stillman B (1998) The DNA replication fork in eukaryotic cells. *Annu Rev Biochem* **67**: 721–751

Walter J, Newport J (2000) Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol Cell* **5**: 617–627

Wang X, Zou L, Zheng HY, Wei QY, Elledge SJ, Li L (2003) Genomic instability and endoreduplication triggered by RAD17 deletion. *Genes Dev* **17**: 965–970

Ward IM, Chen J (2001) Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem* **276**: 47759–47762

Ward IM, Minn K, Chen J (2004a) UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress. *J Biol Chem* **279**: 9677–9680

Ward IM, Minn K, Chen J (2004b) UV-induced ATR activation requires replication stress. *J Biol Chem* **279**: 9677–9680

You Z, Ishimi Y, Masai H, Hanaoka F (2002) Roles of Mcm7 and Mcm4 subunits in the DNA helicase activity of the mouse Mcm4/6/7 complex. *J Biol Chem* **277**: 42471–42479

You Z, Komamura Y, Ishimi Y (1999) Biochemical analysis of the intrinsic Mcm4–Mcm6–mcm7 DNA helicase activity. *Mol Cell Biol* **19**: 8003–8015

Zou L, Cortez D, Elledge SJ (2002) Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. *Genes Dev* **16**: 198–208

Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**: 1542–1548

Zou L, Liu D, Elledge SJ (2003) Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci USA* **100**: 13827–13832